METABOLISM AND FUNCTION

IN HONOR OF OTTO MEYERHOF

Marine Biological Laboratory

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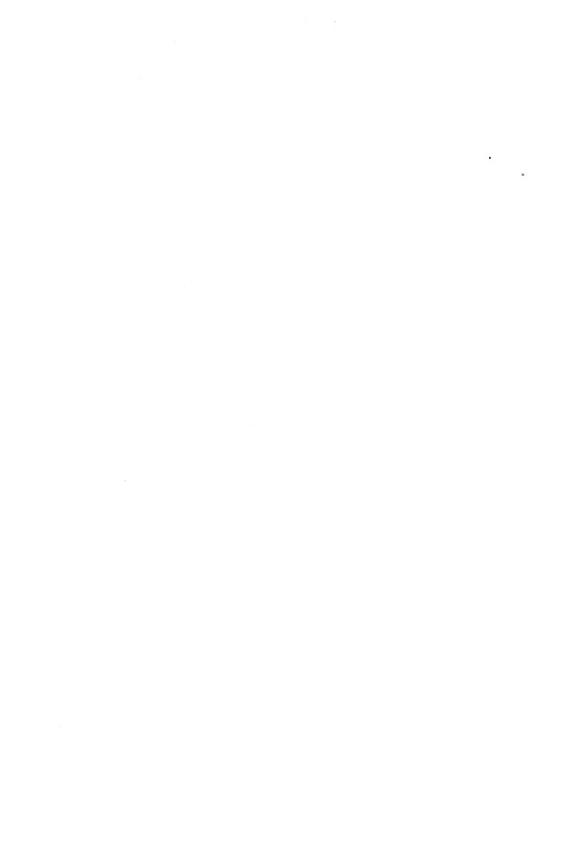


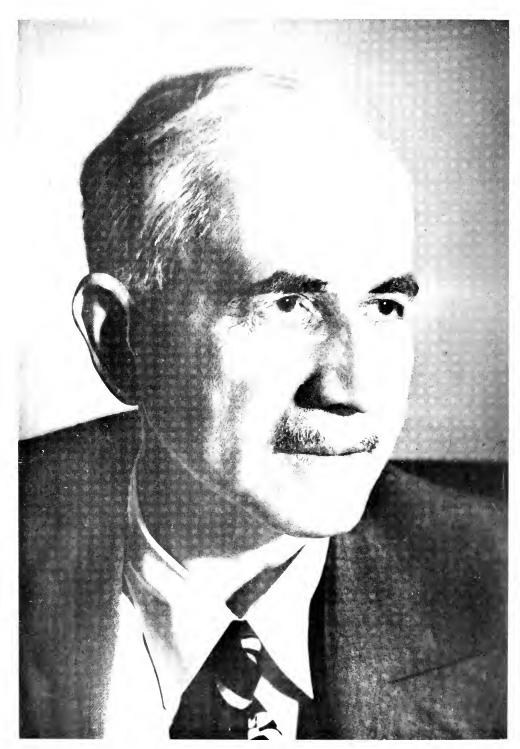


# METABOLISM AND FUNCTION

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OTTO MEYERHOF

METABOLISM AND
FUNCTION

A COLLECTION OF PAPERS DEDICATED TO

### OTTO MEYERHOF

ON THE OCCASION OF HIS 65TH BIRTHDAY

EDITED BY
D. NACHMANSOHN, M.D.



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#### A TRIBUTE ON HIS 65TH BIRTDAY (APRIL 12, 1949)

by

#### DAVID NACHMANSOHN, M.D.

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The scientific work of Otto Meyerhof has profoundly influenced the development of Physiology and Biochemistry of the last three decades. By the originality of his approach, the elegance of his methods, and the wide range of his knowledge and his interests he became a pioneer in many fields.

Otto Meyerhof received his degree of Doctor of Medicine from the University of Heidelberg in 1909. Under the influence of Otto Warburg his interest turned to cellular physiology, especially to aspects concerning energy transformations. The association of these two great scientific figures was extremely fruitful and important for the development of this field.

In 1913 Otto Meyerhof became Privatdozent in Kiel and in 1918 Professor extraordinarius. It was there that Meyerhof started the brilliant work on muscular contraction with which his name will always remain connected and for which he received the Nobel prize in 1923, jointly with A. V. Hill. In 1924 he moved to the Kaiser Wilhelm Institute for Biology in Berlin Dahlem, and in 1929 he became head of the Department of Physiology in the Kaiser Wilhelm Institute for medical research in Heidelberg.

The outstanding feature of Otto Meyerhof's work on muscle is the first really successful attempt to correlate chemical and physical processes of cellular function. He was able to establish such correlations in a great variety of ways and with amazing ingenuity. During these investigations he maintained a continuous exchange of views and information with A. V. Hill. The collaboration between these two men who have maintained a close personal friendship over decades was most fortunate and essential for the development of muscle physiology. These two names will continue to be linked in the History of Science.

In the course of his research on intermediary metabolism in active and resting muscle, Otto Meyerhof discovered many fundamental laws which greatly stimulated the whole of Biochemistry in general. Among his many achievements may be reckoned the clarification of the Pasteur reaction. He showed that oxygen consumption prevented 3 to 6 times the equivalent amount of lactic acid formation in muscle. Otto Wareurg later found the same principle to be true in the glycolysis of tumor cells and Meyerhof in yeast fermentation. Meyerhof's discovery thus proved and extended Pasteur's hypothesis that fermentation is "la vie sans air", i.e., to a certain extent substituted respiration, whereas in the absence of respiration fermentation increases. Pasteur has proposed this assumption but was unable to verify it, because he used cultivated yeast in which respiration is negligible compared with fermentation. This reaction in the

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carbohydrate cycle has been called the Pasteur-Meyerhof reaction. The carbohydrate cycle was the first one to be demonstrated but the idea of cyclic processes in cellular mechanisms has since become more and more generalized. Today it is familiar to every biochemist and an integral part of our thinking.

The discovery of Otto Meyerhof and his students that some phosphorylated compounds are rich in energy led to a revolution, not only of our concepts of muscular contraction, but of the entire significance of celular metabolism. A continuously increasing number of enzymatic reactions are becoming known in which the energy of adenosine triphosphate, the compound isolated by his associate Lohmann, provides the energy for endergonic synthesis reactions. The importance of this discovery for the understanding of cellular mechanisms is generally recognized and can hardly be overestimated.

In 1925 Meyerhof succeeded in extracting the glycolytic enzyme system from muscle, retracing a pathway which Buchner and Harden and Young had explored in yeast. This proved to be a decisive step for the analysis of glycolysis. Meyerhof and his associates were able to reconstruct *in vitro* the main steps of the complicated chain of reactions leading from glycogen to lactic acid. They verified some and extended other parts of the scheme proposed by Gustav Embden in 1932, shortly before his death.

The few examples given may suffice to indicate not only the brilliance but also the wide scope of his achievements. A real appreciation of his work is impossible within a few introductory remarks. Meyerhof has always been driven by the true pioneer spirit. His open and critical mind quickly grasped new developments. When, in 1929, Einar Lundsgaard found that contraction in a monoiodoacetate poisoned muscle occurs without lactic acid formation, Meyerhof rapidly accepted the evidence which was built essentially on his own line of approach. This rapid change of his views shows the strength of his scientific personality and was all the more remarkable since for many years he had vigorously supported the idea that lactic acid formation was the primary step.

After the rise to power of the Nazis, Meyerhof, like other Jewish scientists, had to leave Germany. In 1938 he went to Paris where he was warmly welcomed and well received. By the combined efforts of the late Jean Perrin, Professor René Wurmser and Professor Henri Laugier, he was appointed Director of Research at the University of Paris and was able to continue his research in the Institut de Biologie Physico-Chimique. When the Nazi hordes invaded France, he had to flee again under most difficult circumstances, and came to the United States at the end of 1940. Here he was appointed Research Professor of Physiological Chemistry in the School of Medicine of the University of Pennsylvania, a position he holds at present. In spite of all difficulties his creative spirit is unbroken, as shown by the great number of his publications during the past few years, concerning especially intermediary metabolism, the purification and properties of adenosine triphosphate, the free energy of phosphorylated compounds, and various other subjects.

In spite of his intense scientific activity, Meyerhof's interests have never been limited to science. The extraordinarily wide scope of his nonscientific activities shows best his rich personality. From his student years on he had been not only interested but actively engaged in philosophy. He was closely associated with the Nelson group in Göttingen. He devoted much time to a critical analysis of Goethe's scientific work and presented recently at the Goethe Bicentennial Celebration of the Rudolph Virchow

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Society in New York a profound and most lucid and critical evaluation of Goethe's scientific ideas and concepts, especially the Farbenlehre. He always had and still has a passionate love of art, literature and poetry. His interest in painting has been greatly stimulated by his wife Hedwig who is a painter and actively engaged in teaching the art of painting. No matter which field Meyerhof discusses, it is always a great stimulus and his views show the originality of his ways of thinking and his remarkable gift of integrating a great variety of phenomena.

Otto Meyerhof's 65 birthday offers a happy occasion for his former associates to express their gratitude and for his friends their esteem. The contributions of this anniversary volume are only a very incomplete indication of the influence of his work in so many fields. They are offered as a small tribute to his creative genius.

## PART I

#### MUSCLE

#### A CHALLENGE TO BIOCHEMISTS

by

#### A. V. HILL

Biophysics Research Unit, University College, London (England)

OTTO MEYERHOF has always been betwixt and between: a physiological chemist or a chemical physiologist, perhaps we should call him a "chemiologist". On my shelves are about two hundred of his reprints, his and his colleagues'. The first of these, with its accompanying letter addressing me as "Sehr geehrter Herr Kollege" dated 1911 from Naples, dealt with the heat production of the vital oxidation process in the eggs of marine animals. Next follow papers on the energy exchanges of bacteria, the heat accompanying chemical processes in living cells, the inhibition of enzyme reactions by narcotics (1914). Some time in those apparently peaceful years, before the explosion of 1914, he visited us at Cambridge. Then comes a gap, so far at least as my collection of Otto Meyerhof's reprints is concerned. By 1919 he had moved to Höber's laboratory at Kiel and the long succession of papers began on the respiration, energetics, and chemistry of muscle. And when I say muscle, I mean muscle: living muscle, resting, contracting and recovering from contraction, developing tension and doing work, producing lactic acid and removing it again, using oxygen and glycogen, giving out CO, and heat, all things which living muscles are accustomed to do. And since I too was working on living muscle, we were in frequent communication again, after the five years' gap. In the summer of 1922, following a suggestion to HOPKINS, he visited Cambridge and gave lectures there. I remember "Hoppy" expressing concern lest some anti-German demonstration might take place, but appearing to be satisfied by the comment that if so I should be proud to remove the demonstrator: nothing of course happened. Later, he stayed with me at Manchester and I recall, as an example of his scientific perspicacity, the complete disbelief which he, first of anyone, expressed in experiments he witnessed which six months later were proved to be fraudulent. That was our first reunion after the War, there were many others, in London, Plymouth, Barcelona, Heidelberg, Berlin, Rome and elsewhere. The photograph shows us driving together to Stockholm for the Physiological Congress in 1926.

The results of his researches, and those of his colleagues, are a part of scientific history. They are linked with most that is known of the chemistry of muscle and with much that is established of changes involving phosphate and carbohydrate in the cell. For some years his investigations were concerned mainly with muscle — living muscle: more recently they followed the trend in biochemistry, perhaps even they helped to establish the fashion, of dealing *in vitro* with the enzyme systems of muscle. As late, however, as 1935, he was working on the volume changes of living muscle during contraction and relaxation and relating them to the underlying chemical cause. I read these papers again recently, very carefully, having come to the conclusion that the

reversible part of the volume change is attributable mainly or wholly to pressure set up by contraction. The elegance and clarity of MEYERHOF's work and its description impressed itself again as it had done in earlier days. One might criticize some of the conclusions, but not the methods or results. To read these papers once more was a sudden pleasure, after so many in which one could not be sure what an author had really done!





My last reprint from Heidelberg is dated 1938. Perhaps if Hitler had not driven him from the beautiful Institute and the excellent colleagues and facilities he had there, the succession of papers on muscle—living muscle—might have continued. Alas that they could not! This paper, however, is to challenge him and his disciples to make a few more chemical investigations on living muscle, to see how far the chemistry *in vitro* of muscle extracts can be fitted to the physical facts of muscular contraction.

It is customary for biochemists (e.g., Baldwin, p. 341) to describe "The probable course of events in normal muscular contraction" is some such terms as these:

\*References p. 11.

"On the arrival of a nerve impulse, ATP is broken down, giving rise to ADP and inorganic phosphate, furnishing at the same time the contraction energy. The ADP is promptly converted again into ATP at the expense of phosphagen and no change in the ATP content of the muscle can be detected..." Others suppose that contraction is associated with the formation of myosin — ATP and that ATP is broken down in relaxation. By Sandow<sup>2</sup> a slight initial lengthening (in a muscle under tension) after a stimulus ("latency relaxation") is attributed to the formation of a complex between activated myosin and ATP. Most of this is pure speculation, without direct experimental evidence. Unlike Mr. Stalin (HISTORICUS<sup>3</sup>) I have no general theory of revolutions, but I did once write an article (1932), which I think is still worth reading, on "The Revolution in Muscle Physiology"4. That was after phosphagen had deposed lactic acid from pride of place as the chief chemical agent in contraction. At that date one could write: "On stimulation, phosphagen breaks down . . .: this is the primary change by which energy is set free". Only four years earlier RITCHIE<sup>5</sup> wrote: "On stimulation of a muscle fibre the wave of excitation passes down it; by increasing the permeability of a membrane or by some other means it causes the liberation of lactic acid from a carbohydrate source. The liberated hydrogen ions neutralize the negative charge on a surface of protein, MEYERHOF's Verkürzungsort . . . and thereby alter the type of structure, the area of surface, and the mechanical constants. This will be the fundamental change." In the lactic acid era the evidence that the formation of lactic acid was the cause and provided the energy for contraction seemed pretty good. In the phosphagen era a similar attribution to phosphagen appeared even better justified. Now, in the adenosine triphosphate era lactic acid and phosphagen have been relegated to recovery and ATP takes their place. Those of us who have lived through two revolutions are wondering whether and when the third is coming.

It may very well be the case, and none will be happier than I to be quit of revolutions, that the breakdown of ATP really is responsible for contraction or relaxation: but in fact there is no direct evidence that it is. Indeed, no change in the ATP has ever been found in living muscle except in extreme exhaustion, verging on rigor. This is explained by supposing that as soon as ATP is broken down into ADP and phosphate it is promptly restored in the so-called "Lohmann reaction" at the expense of creatine phosphate.

$$ADP + CP \rightarrow ATP + C$$

If this happens after each stimulus, then the smallness of the changes involved and their quickness make it extremely difficult to gain any direct evidence on the subject. In a single twitch, for example, the heat set free is about 3 millicalories per gram, which would correspond to the liberation from ATP of  $2.5 \cdot 10^{-7}$  g molecule of phosphate per gram of muscle. To measure so small a change, reversed within the duration of a single twitch, might well seem an impossible task.

We should not, however, be so satisfied with the explanation of why no change in ATP is ever found in living muscle that we cease to look for it: for another possibility exists. The total energy available from all sources (lactic acid, phosphagen and ATP) for the anaerobic phase of contraction is about I cal/g, corresponding to about 400 twitches. The total energy similarly available after poisoning with iodoacetate (from phosphagen and ATP) is about 0.25 cal/g corresponding to about 100 twitches. From the known amount of ATP present is muscle, the total energy it could provide by breaking

off one phosphate is about 0.05 cal/g, corresponding to about 20 twitches. Is it not possible that as stimulation proceeds a balance is reached at some intermediate level between breakdown and restoration? That is the case with phosphagen and lactic acid; in a muscle steadily stimulated (in the presence of oxygen) a certain amount of phosphagen is broken down, a certain amount of lactic is formed, and a steady level is reached between breakdown and recovery. At a still earlier stage one might expect steady stimulation to provide at least a temporary balance between ATP breakdown and restoration.

In frogs' muscles at 20° C, if ATP were the only source of energy a maximal tetanus would lead to its complete breakdown in about 0.5 sec. The suggested balance, if it occurred, would presumably be reached within that time, and when the stimulus ended restoration of the ATP might be completed within another 0.5 sec. The times involved are far too short for chemical manipulation: but biochemists need not be disheartened, frogs' and rabbits' muscles are singularly ill-suited to the enquiry, they are much too quick, why not use muscles which contract more slowly? The muscles of the Mediterranean land tortoise, Testudo graeca, commonly imported before the War into England and sold on barrows for 1/- in London streets, take about fifteen times as long to contract as those of a frog and their speed can be further reduced about nine times by lowering the temperature from 20° C to 0° C, or about five times by lowering it to 5° C. This means that the time available for chemical manipulation can be reckoned in large fractions of a minute instead of fractions of a second. Provided, therefore, that the chemical technique is capable of determining a substantial part of the total ATP with reasonable accuracy, the time involved can be made so long that sufficient resolution ought easily to be obtained.

The experiment ought certainly to be made and nobody could make it better than Otto Meyerhof — for he knows how to handle living muscles. The result may not be unequivocal — but it very well may. If no change in ATP is found, but only a change in phosphagen, the status quo remains and we can all believe what we like, provided it is consistent with the physical facts described below. But suppose it is found that ATP is broken down at a rate decreasing from the start, reaching a steady concentration after half a minute's stimulation (corresponding to half a second in a frog's muscle at 20° C) and is restored to its original level after (say) a further half minute of rest and recovery. Then at least we can be assured that ATP is really concerned either with the contractile process itself, or with the very early stages of recovery. There are other possibilities and, without trying, it is useless to speculate too much. A German clinician is said to have remarked: "Der Versuch muss gemacht werden und sollte er hundert Bauern kosten". A decision on this important matter is certainly worth a hundred tortoises.

But whatever may be the outcome of this challenge to biochemists, I would invite them also, in their speculations about muscle, to take note of the following facts, all referring to contraction and relaxation, as distinguished from recovery.

- 1. There is no sign of an endothermic process at any stage of contraction or relaxation. If endothermic processes occur they are balanced, or overbalanced, by exothermic ones.
- 2. No heat at all is produced during relaxation, apart from that derived from the degradation of work previously performed during contraction (in raising a load, or in stretching elastic material in series with the muscle). When a muscle relaxes without load or tension, no heat is produced after the contractile phase is over.

- 3. It has been found by quick stretches applied to a muscle shortly after a single shock that the full strength of the contraction, defined as the load which a muscle can just bear without lengthening (and equal to the force of a maximal tetanus) is developed abruptly immediately after the end of the latent period. It is maintained for a time and then declines in "relaxation". If stimulation is continued, each successive shock restores the strength of contraction to its full height.
- 4. Corresponding to (3) there is a "heat of activation" in a twitch, which is independent of all other factors except the fact of stimulation. The heat of activation starts at its maximum rate before any visible sign of contraction occurs, declining to zero at about the moment when the strength of contraction (see 3 above) begins to fall off, i.e., at the end of the contractile phase.
- 5. The "heat of maintenance" in a prolonged contraction is the summated effect of the heat of activation following successive elements of the stimulus. It is greater at first corresponding to the more rapid relaxation after a short tetanus, but after a certain duration of stimulus it becomes constant. It is affected only to a minor extent by the length of the muscle. It is greatly increased by a rise of temperature, corresponding to the more rapid relaxation.
- 6. In twitch and tetanus alike, apart from the heat of activation or the heat of maintenance, energy is given out in two discrete forms, (a) as mechanical work and b) as heat of shortening. The heat of shortening is directly proportional to the change of length over the whole range of shortening, and (for a given change of length) is independent of the work done.
- 7. Apart from heat of activation or heat of maintenance, the rate at which total energy, *i.e.*, heat plus work, is given out, is a linear function of the load throughout a contraction:  $(P+a) dx/dt = b(P_0 P)$

where x is the amount of shortening up to time t, P is the load, dx is the heat of shortening,  $P_0$  is the maximum isometric tension and b is a constant related to the maximum velocity of shortening under zero load.

- 8. The constant a in (7) can be obtained either from thermal measurements or from the form of the characteristic relation between load and velocity of shortening. The agreement is good.
- 9. Relaxation is not an active process. A muscle completely without load or tension does not lengthen again after shortening in response to a stimulus. That its length has really changed and that its fibres or fibrils have not gone into folds is shown by the fact that its latent period is practically the same at a short length as it is at a greater one. If a muscle had to "take up the slack" in fibres or fibrils before its tension could be manifested externally, the latent period would be greatly prolonged.
- 10. Simultaneous with the earliest sign of mechanical activity after a shock is a change of opacity. This is due to an alteration of light scattering (D. K. Hill. 6). The earliest phase has certain characteristics which distinguish it from a later phase which continues into recovery.
- 11. If we can assume that excitation occurs at the surface membrane of a muscle fibre, the propagation inwards of the change there started cannot be due to the diffusion inwards of some substance, e.g., Ca ions or acetyl choline, initiating contraction by its arrival at each point. Diffusion is far too slow. Some chain-reaction started at the surface is required.

Nineteen years ago my colleagues and I found (Hill and Kupalov<sup>7</sup>; Hill and Parkinson<sup>8</sup>) in muscles stimulated to exhaustion in nitrogen, a lowering of vapour pressure considerably too large to be accounted for by chemical changes known to occur, if the precursors of the chemical substances produced were themselves osmotically active. In normal muscles complete exhaustion led to a decrease of vapour pressure corresponding to an increased concentration in the free water of a muscle of 0.12 M. The production of 0.35% lactic acid dissolved in the free water, (taken as 0.77 g per g) of the muscle, would lead to a concentration change of 0.050 M. The liberation of creatine and phosphate by the complete breakdown of phosphagen in amounts equivalent to 65 mg. P/100 g would give 0.054 M. The production of phosphate and adenylic acid from ATP in amounts equivalent to 30 mg P/100 g would give 0.012 M. The total, 0.116 M, is not far from that (0.12 M) calculated from the observed change of vapour pressure. We have assumed, however, that the phosphagen and the ATP were not themselves osmotically active; if they had been the increase would have been 0.031 M less, namely 0.085 M instead of 0.12 M. The vapour pressure measurements were certainly not that much wrong.

Again, in muscles poisoned with iodoacetate complete exhaustion led to a mean decrease of vapour pressure corresponding to an increased concentration of 0.050 M. If phosphagen and ATP breakdown are assumed, as above, to be the only chemical reactions involved, the corresponding change of concentration in the free water of the muscle would be 0.066 M. It is impossible, however, in muscles adequately poisoned to ensure that some preliminary breakdown of phosphagen has not occurred; and if the poisoning is not quite sufficient, there is likely to be some formation of lactic acid. Either cause would tend to make the observed change of vapour pressure smaller than that calculated from the assumed breakdowns. Even so, had the phosphagen and ATP originally been osmotically active, the change calculated from the constituents would have been only 0.035 M, considerably less than the 0.050 M observed.

Unless, therefore, some chemical reactions hitherto unknown occur in a muscle stimulated to exhaustion in nitrogen, we are forced to conclude that phosphagen and ATP are not themselves osmotically active in the normal muscle. This would be the case if they were bound to other molecules and their constituents only became free when they broke down. These older experiments are worth recalling now because they are pertinent to the question of how phosphagen and ATP exist in the living muscle. Looking back at them today I see no reason to question their results. If those are correct, ATP and phosphagen exist in a combined form in muscle, exerting no osmotic pressure on their own account until they are broken down.

The work which an isolated muscle of frog or toad can perform under optimal conditions may be as high as 40% of the total energy given out in the initial process, as distinguished from recovery (Hill). This high efficiency is obtained just the same at o° C as at higher temperatures, and there are no grounds at all for supposing that the nature of contraction is in any way altered, except in speed, by a change of temperature. The muscle twitch is rather stronger at o° C than at 25° C, and quite as efficient. If theory predicts otherwise, so much the worse for the theory. The highest efficiency is obtained with a comparatively large load and slow shortening; under isotonic conditions, with a load about half the maximum which the muscle can lift. In such a contraction the work done is about twice the heat of shortening: two thirds of the total energy set free, in excess of the heat of activation (or maintenance), is external mechanical work.

Under conditions, therefore, of maximum efficiency, the energy is liberated in about the following proportions:

| HEAT OF ACTIVATION | Work | Heat of shortening |
|--------------------|------|--------------------|
| OR MAINTENANCE     |      |                    |
| 40                 | 40   | 20                 |

At the other extreme, with zero load and rapid shortening, the situation may be this:

(The heat of activation is the same in both cases.)

The fact that the external work may be so large a fraction of the whole energy liberated in excess of the activation (or maintenance) heat naturally makes one ask whether the heat of shortening may not itself really be work degraded into heat in overcoming some internal resistance to shortening: in that case energy would be liberated in two forms only, heat of activation (or maintenance) and mechanical work. For two reasons, the supposed internal resistance cannot be of a viscous nature: (1) the heat of shortening is independent of the velocity of shortening, and (2) the heat of shortening per cm is the same over the whole range of possible shortening (if it were due to overcoming viscous resistance it would be inversely proportional to the length). The supposed resistance must be constant, and must reside in lines or filaments parallel to the axis of the muscle, it cannot be a volume effect. An obvious objection to the theory of a constant (e.g., frictional) resistance a parallel to and inherent in the contractile elements is that there should then be a constant difference 2a between the load at which a muscle just shortened and the load at which it just lengthened: experiment showed (Katz<sup>10</sup>) that no such difference exists. The objection would be valid if a muscle were a single contractile element, with a parallel constant resistance. In fact, however, a muscle fibre is very long relative to its thickness, and its diameter is by no means constant throughout its length. There is no reason to suppose that its maximum force is the same everywhere. If not, in an isometric contraction the stronger regions would tend to shorten at the expense of the weaker regions, and the constant resistance would hinder shortening at one point and lengthening at another (possibly a very convenient arrangement in a system of non-uniform strength). With a large number of such elements in series an increase of load would stretch the weaker elements, a decrease of load would allow the stronger elements to shorten; and the difference of load between observable lengthening and shortening would be small. The objection, therefore, is not really valid.

A stronger objection, raised in 1938<sup>11</sup>, is that there are indications that the heat of shortening changes sign when shortening becomes lengthening; and the heat generated in overcoming a frictional resistance does not change sign when the direction of motion is reversed. The difficulty is to get muscles to lengthen reversibly except at very low speeds. Possibly the use of dogfish jaw muscles (Levin and Wyman<sup>12</sup>) which stand stretching well would allow more positive conclusions to be reached. One thing is certain, namely that the work done in making a muscle lengthen does not reappear completely as heat: Some of it is absorbed, presumably, in driving chemical reactions in the endothermic direction. The subject is being investigated afresh by improved methods.

One final word — to continue my challenge to biochemists. Otto Meyerhof's first letter to me, as I wrote at the beginning, came from Naples: all his life he has been ready to vary not only his chemical technique but his biological material. The properties of animals, and of their muscular systems, vary over a very wide range. There is no need to stick to rabbits and frogs. If a problem seems insoluble on one muscle, one should try to define it more precisely to see where the difficulty lies. Discussion with a zoologist, or a visit to a Marine Laboratory, may provide material many times better suited to one's needs. I spent many years trying to measure the heat production of nerve: if I had made the experiment on crabs' nerves instead of frogs' the answer would have come in 1912 instead of 1926. In 1912 it was not possible to define the problem well enough to get a clear direction to non-medullated nerve, but at least one might have taken a chance and not persisted with the frog's sciatic. If one's instruments, or methods, are too slow, one can make them relatively quicker by using slower material — tortoises, toads or even sloths. That means, of course, that biochemists, like biophysicists, must also be biologists (as Meyerhof has always been and as Hopkins was) — but why not?

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#### MUSKELPROTEINE

von

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Es ist wahrscheinlich, dass bei keinem anderen Gewebe Stoffwechsel, Energetik und kolloidaler Feinbau so gut bekannt sind wie beim Skelettmuskel. Es ist sicher, dass bei keinem anderen Gewebe der *Zusammenhang* zwischen diesen Eigenschaften lebender Systeme auch nur annähernd so weit geklärt ist, wie bei der Muskeltätigkeit.

Bei den Muskelproteinen betraf sogar die erste fundamentale Entdeckung gar nicht die Proteine selbst, sondern gerade diesen Zusammenhang: 1922 stellte O. Meyerhof<sup>19</sup> fest, dass etwa <sup>1</sup>/<sub>3</sub> der Wärmeproduktion der Arbeitsphase des Muskels auf der Bindung der H-Ionen der Milchsäure durch die Muskeleiweisskörper beruhe und dass in der Erholungsphase ein entsprechender Betrag der Verbrennungsenergie der Milchsäure verbraucht würde, um die H-Ionen wieder von dem Eiweiss abzulösen.

Man wusste damals fast nichts über Zahl und Art der Muskeleiweisskörper. Von Fürth¹² hatte aus dem Muskelpressaft ein Muskelalbumin isoliert, das Myogen, mit zahlreichen und verwickelten Denaturierungsmechanismen. Er hielt es ausserdem für möglich, dass im Pressaft auch noch ein besonderes Protein vorhanden sei, das er für den Träger der Muskelkontraktion hielt, und für das er den Namen Myosin vorschlug. Er war allerdings nicht sicher, dass dieses Myosin ein Eiweisskörper sui generis sei und nicht ein Denaturierungsprodukt des Myogens. Soweit diese Zweifel die Anwesenheit des Myosin im Muskelpressaft betrafen, waren sie berechtigt: denn das kontraktile Protein geht nicht in den Muskelpressaft über³8, ²7.

Die Entdeckung Meyerhof's war trotz oder gerade wegen dieser Unsicherheiten ausserordentlich folgenreich. Denn Meyerhof hatte schon selbst gleich in seiner ersten Originalarbeit festgestellt, dass die angeführten Wärmetönungen bei H'-Bindung und H'-Abgabe offenbar bei allen Proteinen in neutralem und alkalischem Milieu auftreten. Nun verläuft aber H'-Bindung und H'-Abgabe durch Carboxylgruppen in der Regel athermisch, durch organische basische Gruppen aber mit solchen Wärmetönungen, wie sie Meyerhof gefunden hatte. Das würde bedeuten, dass Eiweisskörper auf der alkalischen Seite des isoelektrischen Punktes nicht, wie man bis dahin geglaubt hatte, mit ihren Carboxylgruppen, sondern mit ihren basischen Gruppen puffern, oder mit anderen Worten, dass isoelektrische Eiweissteilchen nicht Moleküle sondern Zwitterionen sind. Und so wurde die Meyerhof'sche Entdeckung am Muskel zu einem fundamentalen Argument der Zwitterionentheorie der Aminosäuren und Eiweisskörper<sup>35</sup>.

Da aber im übrigen grosse Unterschiede in der Wärmetönung der H'-Bindung nicht nur zwischen Carboxylgruppen und basischen Gruppen bestehen, sondern auch zwischen den verschiedenen basischen Gruppen unter sich, wirkte die Меуевноб-Entdeckung noch weiter. Jesse P. Greenstein<sup>15</sup> mass diese Wärmetönungen an den trivalenten Aminosäuren und ihren Peptiden. Er schuf damit die Unterlagen, um die Wärmetönung der Eiweisspufferung in verschiedenen p<sub>H</sub>-Bereichen auszuwerten für die Beantwortung der Frage, welche der ionogenen Gruppen in dem jeweiligen p<sub>H</sub>-Bereich Träger der Pufferung wären<sup>39</sup>. Wenn wir heute am intakten Proteinteilchen Zahl und Dissoziationsbereich der einzelnen ionogenen Gruppen weitgehend kennen, so ist das u.a. eine Folge der Meyerhof'schen Muskelstudien.

Π

Das fehlende systematische Wissen um die Zahl und Art der Muskeleiweisskörper wurde in den nächsten 15 Jahren nach Meyerhof's Entdeckung im Groben nachgeholt. Der Stand dieses Wissens wurde 1934<sup>37</sup> erschöpfend und 1939<sup>38</sup> in den wesentlichsten Zügen zusammenfassend dargestellt. Das, was wir heute wissen, ist — unter Ausschluss der elektrischen Ladungsverhältnisse der Proteine und der optischen Resultate\* aus Tabelle I zu ersehen.

Für die Beurteilung der Bedeutung der Hauptfraktionen der Muskelproteine gelten folgende Überlegungen: die Myogenfraktion umfasst nicht nur 20% der Muskeleiweisskörper, sondern sie beansprucht auch 20% des Faservolumens. 80% des Faservolumens sind für Myogen "nichtlösender Raum" <sup>16</sup>. Das bedeutet, dass Myogen dort, wo es im Muskel ist, sich in einer Konzentration von 20% vorfindet. Ebenso stimmt der kolloidosmotische Druck der Muskelfaser recht gut mit dem osmotischen Druck einer 20% igen Myogenlösung überein<sup>10, 37</sup>. Da Myogen unter physiologischen Bedingungen > 30% löslich ist, ist das Myogen also auch im Muskel selbst gelöst. Da diese Myogenlösung im Muskel noch nicht einmal den Raum des Sarkoplasmas vollständig beanspruchen würde, liegt es nahe wenigstens den Hauptteil der Fraktion (Myogen B) als Bestandteil des Sarkoplasma anzusehen.

Die Stromafraktion umfasst — nach mikroskopischer Beobachtung an der erschöpfend extrahierten Muskelfaser—bindegewebige Anteile, Sarkolemm und vielleicht noch einige weitere nicht oder nicht wesentlich doppelbrechende unlösliche Strukturanteile.

Über die Bedeutung der Globulin X-Fraktion sind Aussagen noch nicht möglich.

Die Stellung der Myosinfraktion in der Muskelfaser wurde bisher auf Grund folgender Tatsachen beurteilt: die Eigendoppelbrechung der Faser beträgt ~ 40 (44%) der Eigendoppelbrechung des Myosinfadens gleicher Eiweisskonzentration³6, ²², während die Stäbchendoppelbrechung sogar genau 40% der Stäbchendoppelbrechung des Fadens ausmacht³6, ²². Die Stäbchendoppelbrechung des Fadens ist dabei auch quantitativ die Doppelbrechung eines idealen Wiener'schen Stäbchenmischkörpers. Da ferner auch 40% der Muskeleiweisskörper der Myosinfraktion angehören, wurde gefolgert, dass die gesamte Doppelbrechung des Muskels ausschliesslich auf der Doppelbrechung der Myosinfraktion beruhe und dass auch im Muskel die Myosin- (Aktomyosin) Stäbchen streng achsenparallel angeordnet sind. Da ferner das Volumen der A-Abschnitte auf etwa 40% des Faservolumens geschätzt werden muss, ergab sich als zweiter Schluss, dass wahrscheinlich alles Aktomyosin sich in den doppelbrechenden Abschnitten befindet³6. Daraus und aus der weiteren Tatsache, dass das Aktomyosin der Träger der röntgenoptischen Phänomene des Muskels und ihrer Veränderung bei der Kontraktion ist³, ¹, ergab sich schliesslich, dass Aktomyosin offenbar das kontraktile Protein sei.

Nun fanden Wolpers<sup>42</sup>, sowie Schmitt und Mitarbeiter<sup>28</sup>, dass elektronenmikroskopische Eiweissfäden von einer Dicke von 50 bis 250 Å in gleicher Dichte den  $\Lambda$ - und

<sup>\*</sup> Röntgen-, Polarisations- und Elektronenoptik, sowie Streuung des sichtbare Lichtes.

TABELLE

| Protein-Fraktion                      | Anteil am<br>Gesamtprotein | Name des einzelnen<br>Proteins                                   | Löslich zwischen<br>p <sub>H</sub> 6 u. 7 bei                                                         | $\frac{\eta' - 1}{c} \\ (c = 0) (G^* = 400)$ |  |
|---------------------------------------|----------------------------|------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|----------------------------------------------|--|
| Albumin <sup>12</sup>                 | 20 % 20                    | Myogen B (80% der<br>Fraktion)                                   | o bis $6\mu^{37}$                                                                                     | 0.04 <sup>37</sup>                           |  |
|                                       |                            | Myogen A <sup>4</sup> = Aldolase <sup>7</sup> (20% der Fraktion) |                                                                                                       | _                                            |  |
| Globulin X <sup>20</sup>              | 20 % 20                    | nicht bearbeitet                                                 | 0.005 $\mu^{20}$ bis ?                                                                                | 0.14 <sup>37</sup>                           |  |
|                                       |                            | L-Myosin <sup>31</sup>                                           | 0.05 $\mu$ bei p <sub>H</sub> 6.7 <sup>20, 34</sup> bis 5.7 $\mu$ bei p <sub>H</sub> 5.5 <sup>9</sup> | 2.2 <sup>26</sup>                            |  |
| Myosin <sup>10</sup> a                | 40%20                      | Aktomyosine <sup>32</sup>                                        | o.3 $\mu$ bei p <sub>H</sub> 6.7 <sup>26</sup> bis 3.3 $\mu$ bei p <sub>H</sub> 5.5 <sup>9</sup>      | 3 bis 4.5 <sup>20</sup>                      |  |
|                                       |                            | Aktin (aktiv) <sup>32</sup>                                      | o bis 2 $\mu^{32}$                                                                                    | 2.3 bis 3.2 <sup>26</sup>                    |  |
|                                       |                            | Aktin (inaktiv) <sup>32</sup>                                    | $\circ~\mu^{32}$                                                                                      | 0.0140                                       |  |
| Stroma                                | 20 % 20                    | nicht bearbeitet                                                 | nicht löslich                                                                                         |                                              |  |
| Summe                                 | 100%                       |                                                                  |                                                                                                       |                                              |  |
| Proteine unbekannter<br>Zugehörigkeit | 6%2                        | Tropomyosin <sup>2</sup>                                         | o.1 bis 7 $\mu^2$                                                                                     |                                              |  |

<sup>\*</sup> G = Gefälle

den I-Abschnitt durchziehen. Infolgedessen sollte im I-Abschnitt etwa dieselbe positive Stäbchen-Do\* auftreten wie im A-Abschnitt, d.h. etwa 70% der Gesamt-Do des A-Abschnittes²². Diese Do der I-Bande aber fehlt! Der Widerspruch würde sich lösen, wenn man annimmt, dass die elektronenmikroskopischen Fadenmizellen des I-Abschnittes eine negative Eigen-Do besitzen, die die positive Stäbchen-Do ungefähr kompensiert. Tatsächlich fanden SZENT-GYÖRGYI und seine Schüler¹³, dass gerade in der I-Bande ein Protein—von ihnen N-Protein genannt—vorhanden ist von beträchtlicher negativer Eigen-Do und positiver Stäbchen-Do. Beim Brechungsindex des Wassers wird die positive Stäbchen-Do durch die negative Eigen-Do vollständig aufgehoben. Die Gesamt-Do der I-Bande wird nach erschöpfender Extraktion des Muskels sogar ganz schwach negativ\*\*. Es bleibt zu prüfen, ob die Menge des N-Proteins reicht, um aus

<sup>\*</sup> Do = Doppelbrechung.

<sup>\*\*</sup> SZENT-GYÖRGYI<sup>34</sup> nimmt an, dass sich in der I-Bande die gleichen Mengen und Strukturen an Aktomyosin fänden wie in der A-Bande und infolgedessen die gleiche positive Gesamt-Do wie dort — nur maskiert durch eine entsprechende hohe negative Do des N-Protein. Er übersieht dabei aber, dass die von ihm angeführte negative Gesamt-Do des N-Protein nur bei Einbettung in Medien

| $S_{20} \cdot 10^{13}$ (c = 0)           | (c = o)                                | М                                                                                                                   | $\frac{1}{\zeta}$ osmot.          | $\underset{\text{aus }D_{20}}{\overset{\mathtt{I}}{\varsigma}}\text{u. } s_{20}$ |
|------------------------------------------|----------------------------------------|---------------------------------------------------------------------------------------------------------------------|-----------------------------------|----------------------------------------------------------------------------------|
| 6.4**                                    | _                                      | 81 000 osmot. <sup>31</sup> <i>a</i>                                                                                | 3.0 aus M ${\bf u}.~{\bf s_{20}}$ | _                                                                                |
| 7.8614                                   | 4·7 <sup>814</sup>                     | $1.5\cdot 10^5\mathrm{aus}\mathrm{s}_{20}\mathrm{u}.\mathrm{D}_{20}$                                                |                                   | 5.5                                                                              |
|                                          | _                                      | _                                                                                                                   | _                                 | ,                                                                                |
| 7.126a, 30, 23                           | 0.9 $^{26a}$ 0.5 $^{33}$ , $^{40}$ (?) | 0.84·10 <sup>6</sup> (26a)<br>1 bis 1.5·10 <sup>6</sup> (?) <sup>33</sup>                                           | 1 28<br>—                         | 100.2 aus M u. s <sub>20</sub><br>180(?)                                         |
| 93 bis 280 <sup>40</sup>                 | 0.5**                                  | 14·10 <sup>6</sup> aus s <sub>20</sub> u. D <sub>20</sub>                                                           | _                                 |                                                                                  |
| $64^{40}$ bei c = 0.1%                   |                                        |                                                                                                                     |                                   |                                                                                  |
| $3.7^{**40}$ bei c = $0.24 \frac{\%}{0}$ |                                        | 76 000 <sup>33</sup>                                                                                                |                                   |                                                                                  |
|                                          |                                        |                                                                                                                     |                                   |                                                                                  |
| 2.52                                     | 2.72                                   | $\begin{array}{c} 880000\text{smot.}^{2} \\ 93000\text{aus}\text{s}_{20}\text{u.}\text{D}_{20}^{\ \ 2} \end{array}$ | III                               | 56                                                                               |

<sup>\*\*</sup> Einzelne Versuchsreihe

ihm die elektronenmikroskopischen Fadenmizellen des I-Abschnittes aufzubauen. Die Tatsache, dass die Gesamt-Do der I-Bande durch erschöpfende Extraktion schwach negativ wird, deutet daraufhin, dass sich in der I-Bande neben dem N-Protein noch ein wenig extrahierbares Protein mit positiver Do findet. Man könnte dabei an Tropomyosin² (6% des Muskeleiweiss) oder auch an einen kleinen Teil des Aktomyosins denken. Die neuen Entdeckungen scheinen eher das Rätsel der Struktur der I-Bande der Lösung näher zu führen als zu neuen Annahmen über den Auf bau des A-Abschnittes zu nötigen. Die kontraktilen A-Banden dürften zu ihrem Auf bau den Hauptteil des Aktomyosins verbrauchen, und das Aktomyosin dürfte also das kontraktile Protein sein.

#### H

1930 hatte Deuticke<sup>8</sup> gefunden, dass bei p<sub>H</sub> 7 die Löslichkeit der Muskeleiweiss-

von hohem Brechungsindex auftritt (Xylol-Canadabalsam). Denn nur hier ist die hohe positive Stäbchen-Do des N-Protein selbst infolge des geringen Brechungsunterschiedes zwischen Eiweissfädchen und Einbettungsmedium weitgehend verschwunden.

körper als Folge excessiven anaeroben Stoffwechsels (Ermüdung, Totenstarre, längere Auf bewahrung von Muskelbrei) deutlich abnimmt. 1933 fanden Meyer und Weber<sup>20</sup>, dass bei 24 stündiger Auf bewahrung von Muskelbrei die Löslichkeit der Myosinfraktion fast völlig schwindet. 1938 zeigten Kamp<sup>17</sup> und Weber<sup>38</sup> am lebenden Kaninchen, dass die Löslichkeitsminderung durch Ermüdung in Sekunden und Minuten mit der Erholung wieder verschwindet. Sie zeigten ferner am Frosch, dass diese Löslichkeitsminderung ausserordentlich viel schneller auftritt, wenn dem Muskel durch Halogenacetat die Milchsäurebildung unmöglich gemacht ist. Die Löslichkeitsminderung beruht also offenbar auf einem Stoffwechselvorgang, der durch die Bildung der Milchsäure rückgängig gemacht wird. Da die Löslichkeitsänderungen durch Zusatz von Kreatin, Kreatinphosphat und Adenylsäure nicht beeinflussbar waren, musste es sich um einen sehr frühen Stoffwechselprozess handeln, der zeitlich der Kontraktion nahe steht. Und schliesslich ergab sich, dass allein die kontraktile Eiweissfraktion, die Myosinfraktion, durch diesen Stoffwechselvorgang reversibel in ihrer Löslichkeit geändert wird.

1939 entdeckten Engelhardt und Ljubimova<sup>11</sup>, dass zwischen Myosinfraktion und Adenosintriphosphat-(ATP)-spaltung enge Beziehungen bestehen: der Elastizitätsmodul von Myosinfäden sinkt bei ATP-Gegenwart ab, und das ATP wird gleichzeitig vom Myosin gespalten. Diese Befunde wurden 1941 von Needham und Mitarbeitern<sup>21</sup> erweitert: auch die Viskosität ( $\eta'$ ) und die Strömungsdoppelbrechung (DRF) sinken unter ATP reversibel ab. — 1942 gelang Schramm und Weber<sup>31</sup> mit der Ultrazentrifuge die Auflösung der Myosinfraktion und ihre Trennung in mehrere Komponenten: eine langsam sedimentierende Komponente (L-Myosin) und mehrere schnell sedimentierende Komponenten (S-Myosin).

Alle diese verschiedenen Linien der Forschung vereinigten sich 1942 in den sensationellen und bedeutenden Ergebnissen von Szent-Györgyi und seinen Schülern<sup>32</sup> und in den Untersuchungen anderer Autoren, die von diesen Ergebnissen ihren Ausgang nahmen. Szent-Györgyi bestätigte die Befunde der Needham-Gruppe—übrigens ohne sie zu kennen—und erweiterte sie dahin, dass durch ATP auch noch die Lichtstreuung und die Löslichkeit reversibel beeinflusst würden — aber nicht der Myosinfraktion sondern nur einer Komponente, des Aktomyosin. Damit war auch der Befund Weber UND SCHRAMM bestätigt, dass die Myosinfraktion aus mehreren Komponenten besteht. Einen gewissen Abschluss fand die Erklärung aller dieser Phänomene durch den Beweis, dass die Aktomyosinkomponente eine Verbindung zweier Fadenproteine, des Aktin und des Myosin, darstellt, die bei Gegenwart von ATP unter Änderung aller der Eigenschaften dissoziiert, deren ATP-Abhängigkeit oben angeführt wurde. Schliesslich ergaben die Untersuchungen der Szegeder Schule auch noch, dass die Extrahierbarkeit der Myosinfraktion auf hört, sobald die gesamte ATP des Muskels gespalten ist. Damit war der Deuticke-Kamp-Effekt auf Bildung des schwer löslichen Aktomyosin durch ATP-Mangel zurückgeführt.

IV

Der Versuch, die Komponenten der Myosinfraktion zu trennen, führte zunächst nur zu einer Reindarstellung des L-Myosin (Schramm und Weber³) bezw. des von Szent-Györgyi³¹ so genannten "Myosin" (kristallisiertes Myosin). Es ist aber leicht²⁶, L-Myosin und S-Myosine sauber quantitativ von einander zu trennen: ein Muskelextrakt von 0.6  $\mu$  (0.3 m KCl + 0.15 m Standartphosphat nach Szent-Györgyi) wird auf Literatur S. 24.

0.04  $\mu$  verdünnt und die Myosinfraktion abzentrifugiert. Aus der ATP freien Lösung des Niederschlages fallen dann bei  $p_H$  6.8 alle S-Myosine geschlossen durch Verdünnung auf 0.28 bis 0.3  $\mu$  aus. Die überstehende Lösung enthält nur noch L-Myosin, das bei 0.05  $\mu$  als Gel von 0.5 bis 1% und bei 0.03  $\mu$  als Gel von  $\geq$  2% quantitativ ausfällt. Der lockere Niederschlag der S-Myosine schliesst etwas gelöstes L-Myosin ein, dass durch weitere Umfällungen entfernt werden kann\*. Nach solcher Trennung ist es leicht zu beweisen, dass L-Myosin mit dem Myosin Szent-Györgyi's und die S-Myosine mit seinem Aktomyosin identisch sind.

Werden Extrakte in dieser Weise aufgeteilt, so ordnen sich die Sedimentationskonstanten von mehr als 10 Präparaten des L-Myosin in schwacher und geradliniger

Abhängigkeit von der Eiweisskonzentration zur Kurve I der Fig. I. Auf dieser Kurve liegen auch unsere Werte für "kristallisiertes" Myosin. Bei c = 0 beträgt  $s_{20}$  7.1.

Die Sedimentationskonstanten der S-Myosine sind viel grösser, hängen von der Eiweisskonzentration viel stärker und ausserdem nicht geradlinig ab (vergl. Kurven 2 und 3 der Fig. 1). Bei den S-Myosinen ist vielmehr 1/s der Konzentration geradlinig proportional nach der Formel

$$\frac{I}{S} = \frac{I}{S_{(c=o)}} + K \cdot c$$

 $s_{20}$  (c = 0) ist bei verschiedenen Präparaten der S-Myosine sehr verschieden, K dagegen weniger: so hat  $s_{20}$  (c = 0) in Kurve 2 den Wert 93, in Kurve 3 den Wert 280, während  $K = \Delta_{\tilde{s}/c}^1$  für die Kurven 2 und 3 den Wert 8.8 bezw. 8.2 besitzt. Wenn das immer so ist,

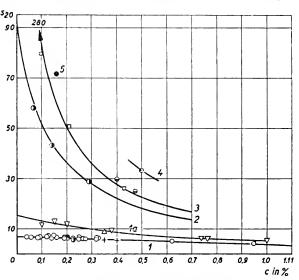


Fig. 1. Sedimentationskonstante
Kurve 1: L-Myosin ○ ○ rein durch fraktionierte Umfällung, ++ rein durch Kristallisation, ⑤ ⑤ aus
Aktomyosin der Kurven 2 und 4 durch ATP; Kurve 2,
3, 4: Aktomyosine ⑥ □ ⑥ aus Muskelextrakt isoliert
durch fraktionierte Umfällung; • 5 aus L-Myosin der
Kurve 1 durch Aktin; ⑥ durch Rückbildung aus ⑥ der
Kurve 1 bei Aufspaltung der ATP; Kurve 1a: Denaturiertes L-Myosin ▽ rein, △ zu 50% gemischt mit
undenaturiertem L-Myosin.

so würde es bedeuten, dass die Wechselwirkung der Einzelteilchen bei den verschiedenen Aktomyosinen annähernd gleich ist, während Gestalt und Grösse der Teilchen von einem Aktomyosin zum anderen sehr verschieden sein können. Denn K charakterisiert die Wechselwirkung,  $s_{20}$  (c = 0) dagegen das Einzelteilchen. Es wurden unter 40 Sedimentationskonstanten von S-Myosinen keine Werte gefunden, die tiefer lagen als die Werte von Kurve 2. In Abbildung 1 sind nur solche Konstanten eingezeichnet, die an einheitlichen Präparaten gefunden wurden. Reine Präparate von S-Myosinen enthalten nämlich häufig 2 S-Myosine mit scharf unterschiedlichen Sedimentationskonstanten.

Werden S-Myosine mit einer genügenden Menge ATP versetzt, so fallen ihre

<sup>\*</sup> Die Trennung bei einem o.6 m KCl-Extrakt ist kurz beschrieben im FIAT-Review40.

Sedimentationskonstanten reversibel auf die Werte des L-Myosin (vergl. Punkt ) der Kurven 2 und 1 und Punkt () der Kurve 4 der Fig. 1, siehe ferner Fig. 2).



Fig. 2. a) Aktomyosin der Kurve 4 (Fig. 1); b) nach ATP-Zusatz

Wird L-Myosin mit einer genügenden Menge Aktin versetzt, so verschwindet seine Sedimentationskonstante und es tritt dafür die Sedimentationskonstante eines S-Myosin auf (vergl. Punkt ◆ Kurve I mit Punkt ◆ 5 der Fig. I). Wird zu wenig Aktin hinzugesetzt, so tritt ebenfalls die Sedimentationskonstante eines Aktomyosin auf, aber es bleibt ausserdem ein Teil des L-Myosin erhalten.

Die Präparate des langsam sedimentierenden Myosin und des Myosin nach SZENT-GYÖRGYI haben eine niedrige, ATP-unempfindliche Viskosität, die vom Gefälle erst bei sehr niedrigen Werten stärker abhängt; die Viskosität der S-Myosine ist für jedes Präparat verschieden, sehr viel höher, stärker vom Gefälle abhängig und fällt auf ATP-Zusatz ungefähr auf den Wert des L-Myosin (vergl. die Kurven 1, 2 und 3 der Fig. 1 und 3)\*.

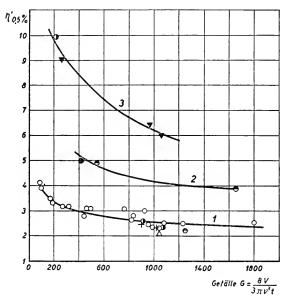


Fig. 3. Viskositäten. Kurve 1: L-Myosin, Kurve 2 und 3: Aktomyosine, die Zeichen für die einzelnen Versuchspunkte haben dieselbe Bedeutung wie in Fig. 1.

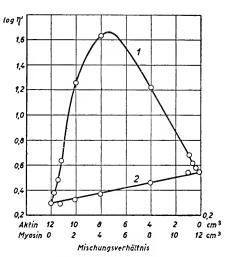


Fig. 4. Viskositäten künstlicher Aktomyosine, Kurve 1 vor ATP-Zusatz, Kurve 2 nach ATP-Zusatz; Abszisse Mischungsverhältnis von Aktin- und L-Myosinlösung in ml, Ordinate log  $\eta'$ . Aktin 0.385% ig, L-Myosin 0.701% ig.

Genau genommen fällt die Viskosität von Aktomyosinen durch ATP auf einen Wert, der sich

<sup>\*</sup> Vergleicht man nur die Aktomyosine unter sich, so wachsen Viskosität und Sedimentationskonstante keineswegs parallel (vergl. Punkt 🌎 der Kurve 4 der Fig. 1 mit Punkt 🔵 der Kurve 2 der Fig. 3). Das ist nicht wunderbar: denn das Achsenverhältnis wirkt auf beide Phänomene entgegengesetzt.

aus einem Beitrag des freien L-Myosin und freien Aktin des Komplexes zusammensetzt — und zwar so, dass sich unter ATP der log  $\eta'$  der L-Myosin- und der Aktinkomponente addiert und nicht etwa die beiden  $\eta'$  Werte selbst (Fig. 4)<sup>18</sup>. Da aber im ATP-Versuch nach Szent-Györgyı immer die Viskosität auf die Gesamteiweisskonzentration (L-Myosin + Aktin) bezogen wird und da ferner  $\eta'$  für Aktin- und Myosinlösungen gleicher Konzentration sehr ähnlich ist, fällt der Unterschied nicht sehr auf (s. u.).

Von der Konzentration hängen die Viskositäten aller Myosinkomponenten und des aktiven Aktin nach der Arrhenius-Formel log  $\eta' = \text{K} \cdot \text{c}$  ab<sup>\*</sup>. Bei Gefälle 1000 beträgt der K-Wert für L-Myosin 0.9 und streut für aktives Aktin zwischen 0.9 und 1.3.

Die häufig auftretenden<sup>30</sup> Sedimentationskonstanten der Kurve 1a der Fig. 1 stammen von einheitlichem, denaturierten L-Myosin: einheitliche Präparate mit diesen Sedimentationskonstanten und Mischungen solcher Präparate mit L-Myosin geben die niedrige Viskosität des L-Myosin und sind ATP-unempfindlich (vergl. Kurve 1a der

Fig. 1 mit Kurve 1 der Fig. 3)30. Die z Komponente mit  $s_{20}$  (c = 0) = 15 ist 90 also kein Akto- oder S-Myosin. Und sie entsteht aus L-Myosin im Laufe der Zeit und der Umfällungen (vergl. Fig. 5). 70 Die "Kristallisation" begünstigt durch ihre höhere Dauer diesen Vorgang mehr als die oben beschriebene Abtrennung des L-Myosin durch fraktionierte Umfällung (vergl. Fig. 5b und c). Die 40 Denaturierung vollzieht sich offenbar in scharfen Stufen. Zwischenwerte zwischen den Kurven I und Ia wurden nie beobachtet. Mit fortschreitender Denaturierung wächst nur der Anteil der denaturierten Komponente (s20 (c = o) = 15) auf Kosten des ursprünglichen L-Myosin (vergl. d und e der Fig. 5).

Während der letzten Jahre wurden im Laboratorium von SVEDBERG gleichzeitig mit unseren Untersuchungen die Sedimentationskonstanten der unfraktionierten Myosinlösungen untersucht<sup>30</sup>. Die Ergebnisse stimmen experimentell mit den hier ange-

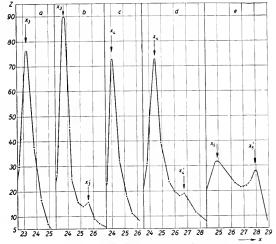


Fig. 5. Sedimentationsgradienten.

Linker Gipfel = L-Myosin mit der Sedimentationsgeschwindigkeit der Kurve I der Fig. I, rechter Gipfel (in b, d und e) denaturiertes L-Myosin mit der Sedimentationsgeschwindigkeit der Kurve Ia der Fig. I. a) = I × "kristallisiert" 4 Tage p.m., b) = 2 × "kristallisiert" 8 Tage p. m., c) = 2 × fraktioniert umgefällt 4 Tage p. m., d) = ebenso, aber 20 Tage p. m., e) = 2 × "kristallisiert", I × umgefällt, 9 Tage p.m.

gebenen Werten für die gereinigten einheitlichen Komponenten überein. Dagegen sind die Sedimentationskonstanten bei den schneller sedimentierenden Komponenten etwas anders auf c=o extrapoliert. Dies beruht darauf, dass die Extrapolationsstrecke wesentlich grösser ist als bei uns und dass s und nicht 1/s geradlinig extrapoliert wurde. So werden die Sedimentationskonstanten der Kurve 1a (Fig. 1) für c=o auf 12 und der Kurve 2 auf 50 extrapoliert statt auf 15 bezw. 93. Für die langsamste Komponente und ebenso für das kristallisierte Myosin nach Szent-Györgyı wird  $s_{20}$  (c=o) mit 7.2 Svedberg. 3 angegeben in guter Übereinstimmung mit unserem Wert von 7.1 Svedberg. Die angegebenen Sedimentationsdaten dürfen also als gesichert angesehen werden.

Fügen wir hinzu, dass der scheinbare Absorptionskoeffizient infolge von Lichtstreuung bei L-Myosinlösungen ∼ 0.1 cm<sup>-1</sup> ist—nach Abzentrifugieren sehr feiner

<sup>\*</sup> Dies gilt im Grunde nur streng, wenn  $\eta'$  aus Messungen mit üblichen Ostwald- oder Ubbelohde-Viskosimetern ohne Hagenbach-Korrektur berechnet wird. Mit Hagenbach-Korrektur hängt log  $\eta'$  nicht mehr ganz geradlinig von der Konzentration ab; die Abhängigkeit folgt dann der Formel von G. V. Schulz und F. Blaschke<sup>18</sup>, <sup>29</sup>.

ungelöster Partikel mit 10000 Touren sogar nur 0.05 cm $^{-1}$ —, während er bei S-Myosinen und künstlichen Aktomyosinen  $\sim$  1 cm $^{-1}$ , so ist damit die Identität von S-Myosinen mit Aktomyosinen und von L-Myosinen mit "Myosin" durch Übereinstimmung in allen Eigenschaften bewiesen $^*$ .

Zu Szent-Györgyi's Anschauungen ergibt sich nur in einem wichtigen Punkt eine Differenz: Aktomyosine sedimentieren mit verschiedenen scharf getrennten Sedimentationsgeschwindigkeiten—häufig sogar in derselben Aktomyosinlösung. Die Aktomyosinbildung aus den beiden Komponenten scheint also in Stufen stattzufinden und nicht gleitend in beliebiger Proportion—genau so wie das L-Myosin in scharf getrennten Sedimentationsstufen denaturiert.

Die spärlichen vorläufigen Angaben über Sedimentation und Viskosität des inaktiven und aktiven Aktin sind aus Tabelle 1 zu ersehen.

V

Werden die zahlreichen L-Myosin-Präparationen der Sedimentationskurve I der

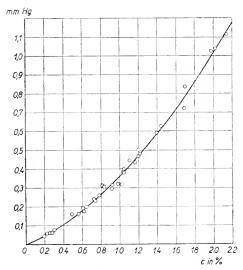


Fig. 6. Osmotischer Druck von L-Myosin

Fig. 1 auf ihren osmotischen Druck untersucht, so steigt der osmotische Druck bis zur Konzentration 2.2% von 0 auf 1.16 mm Hg (Fig. 6)\*\*. Aus der P/c Kurve (Fig. 7) ergibt sich für P/c<sub>lim 0</sub> der Wert 2.05·10<sup>-1</sup>, d.h. ein Teilchengewicht von 840000 (± 33000).

Aus diesem Teilchengewicht und  $s_{20}$  (c = 0) errechnet sich  $D_{20}$  (c = 0) zu  $0.874 \cdot 10^{-7}$ . Der vorläufige Mittelwert unserer direkten Bestimmung ergibt  $D_{20} = 0.9 \cdot 10^{-7***}$ .

Wird aus dem Teilchengewicht und der Sedimentationskonstanten das Achsenverhältnis berechnet, so ergibt es sich zu  $I/\varsigma = 102$ .

Wird das Achsenverhältnis auf Grund der Untersuchungen von G. V. Schulz<sup>28a</sup> über Mischungsentropie und osmotischen Druck berechnet nach der Formel\*\*\*\*

$$q = \frac{4 \varsigma B}{\pi A} \cdot 10^3$$

so ergibt sich das Achsenverhältnis q zu 128.

\*\* Die Methodik der "Messung sehr kleiner osmotischer Drucke" ist von H. Portzehl und H. H. Weber beschrieben<sup>25</sup>.

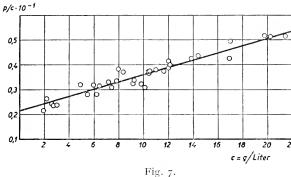
\*\*\*\*  $q = Achsenverhältnis; \varsigma - Dichte des Eiweiss; <math>A = p/c_{tim o}$ , B = Ap/c; c = Gramm/Liter.

<sup>\*</sup> Da der Ausdruck Myosin sehr häufig für die Gesamtfraktion und ihre Lösungen gebraucht wird, erscheint es als eine klare und kurze Bezeichnungsweise, dies weiterhin zu tun, das sogenannte "kristallisierte" Myosin als L-Myosin und die Myosin-Aktin-Komplexe als Aktomyosin zu bezeichnen. Die Ausdrücke Myosin A und B für kurz, bezw. lang extrahierte Gesamtfraktionen würden sich gut in diese Nomenklatur einfügen. Die allgemeine Annahme dieses Vorschlages würde die Verständigung erleichtern.

<sup>\*\*\*</sup> Der in den FIAT-Reviews auf Grund einer einzigen Konzentrationsreihe, die von G. Bergold durchgeführt wurde, angeführte Wert für  $D_{20}$  (c = 0) von  $0.45 \cdot 10^{-7}$  hat sich bei Nachprüfung der Unterlagen als unzuverlässig erwiesen und muss fallen gelassen werden, obwohl er mit den Werten übereinstimmt, die Pedersen²³ auf mündliche Mitteilung von Snellman, Jenow und Erdös angegeben hat.

Bei der ausgezeichneten experimentellen Sicherheit der Sedimentationskonstanten und der Kurve des osmotischen Druckes erscheint das Teilchengewicht ~ 840 000 und

das Achsenverhältnis ~100 recht zuverlässig. Die auf Grund von  $S_{20}$  und  $D_{20}$  früher<sup>40, 23, 33</sup> angegebenen Teilchengewichte scheinen dagegen einer sorgfältigen Überprüfung von D<sub>20</sub> zu bedürfen. Denn die Messungen von D<sub>20</sub> sind nicht nur bisher widerspruchsvoll sondern auch sehr empfindlich gegen Beimengungen langsamer diffundierender Denaturierungsformen des L-Myosin.



Reine Präparate von L-

Myosin scheinen monodispers zu sein. Fig. 8 zeigt ein Sedimentationsdiagramm einer L-Myosinlösung nach Skalenmethode. Berechnet man nach dem Verfahren von Bergolp<sup>3</sup> den Betrag, um den sich die Gradientenkurve vom Zeitpunkt i bis zum Zeitpunkt 10

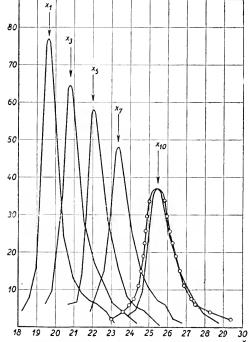
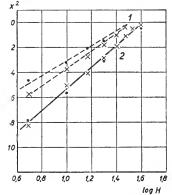


Fig. 8. Sedimentationsgradienten von L-Myosin. - Sedimentationsgradienten gefunden, o-o-o berechnet aus Kurve x<sub>1</sub> und D<sub>20</sub>.

durch Diffusion verbreitert und addiert diesen Betrag zur Breite der Kurve 1, so erhält man die gestrichelte Glockenkurve, die die Gradientenkurve 10 einschliesst. Die gefundene Sedimentation ist also einheitlicher, als sie unter Berücksichtigung des Diffusionseffektes hätte sein dürfen. Der Grund liegt in der Zunahme der Sedimentationsgeschwindigkeit mit abnehmender Konzentration: die durch Diffusion zurückgebliebene Teilchen sedimentieren infolgedessen schneller und die Gradientenkurve wird infolgedessen schmäler und in ihrem vorderen Teil steiler, als sie es auf Grund unbeeinflusster Diffusion geworden wäre. Da aber die Konzentrationsabhängigkeit beim L-Myosin nicht gross ist, und da die experimentelle Gradientenkurve nicht unbeträchtlich schmäler ist, als sie bei reiner Diffusion sein müsste, ist es wahrscheinlich, dass die Sedimentationskonstanten aller einzelnen Myosinteilchen gleich sind.

Dass auch die mechanische Beweglichkeit aller einzelner L-Myosinteilchen auscheinend gleich ist, d.h. die Diffusionskonstante einheitlich ist, geht aus Fig. 9 hervor: Aus der Diffusionsformel lässt sich ableiten, dass bei

einheitlicher Diffusionskonstante das Quadrat der Breite der Diffusionsgradientenkurve geradlinig vom log der Höhe abhängt, in der die Breite gemessen ist. Die Ungestörtheit der Gradientenkurve folgert sich aus ihrer Symmetrie. Die Auswertung zweier beliebiger experimentell gefundener Diffusionsgradientenkurven zeigt (Fig. 9), dass die Quadrate der Breiten rechts und links der Symmetrieachse  $(x_1^2 \text{ und } x_2^2)$  gleich oder fast gleich sind, und



dass beide geradlinig von log H abhängen. Es handelt sich also um störungsfreie Diffusion mit einheitlicher Diffusionskonstante.

Einheitliche Sedimentations- und Diffusionskonstaute aber bedeutet, einheitliche Grösse und einheitliche Gestalt der einzelnen Teilchen des L-Myosin.

Für diese Grösse und Gestalt ergeben sich aus s<sub>20</sub> und Teilchengewicht und unter der plausibelen Annahme eines spezifischen Volumens von 0.75 folgende Masse: 22 bis 23 Å Dicke bei 2200 bis 2400 Å Länge für quadratischen bezw. runden Querschnitt. Vorläufige frühere Angaben<sup>40</sup> sind durch diese Werte überholt.

Das  $\beta$ -Myosin Dubuissons<sup>9</sup> scheint mit dem L-Myosin identisch zu sein. Sollte sich das bestätigen, d.h. sollten die Spuren des  $\gamma$ -Myosin mit dem L-Myosin nichts zu tun haben, so wären alle Teilchen des L-Myosin nicht nur in Grösse und Gestalt, sondern auch in ihrer elektrischen Ladung gleich (vergl. auch Szent-Györgyi<sup>33</sup>).

#### VI

An MEYERHOF'S Entdeckung jenes Zusammenhanges zwischen Kolloidik und Stoffwechsel, der durch die Ionisationswärme der Proteine gegeben ist, schloss sich die erste Periode systematischer Erforschung der Muskelproteine an. Sie führte in der Feststellung der Wechselwirkungen zwischen Adenosintriphosphat und Myosin zu einem neuen Zusammenhang von Stoffwechsel und Eiweisszustand- diesmal sogar Zustand gerade des kontraktilen Proteins. Dieser Zusammenhang gewann eine eindrucksvolle Aktualität dadurch, dass sich Myosinfäden auf ATP-Zusatz bei niedriger Ionenstärke zusammenziehen und bei höherer Ionenstärke wieder ausdehnen (Szent-Györgyi<sup>32, 6</sup>). Von neuem folgte systematische Proteinforschung mit dem Ziel einer verfeinerten Analyse gerade der kontraktilen Eiweissfraktion. Diese Analyse steht noch in ihren Anfängen. Infolgedessen kann der Mechanismus der ATP-Wirkung auf Myosin nur mit Zurückhaltung erörtert werden: in Lösung besteht er zweifellos in einer reversiblen Verminderung der Kohäsionskräfte zwischen Aktin und L-Myosin. Das fadenförmige Gel dagegen wird offenbar (s.o.) nicht von ATP sondern von der Ionenstärke reversibel beeinflusst. ATP scheint nur nötig zu sein, damit der Faden beim Übergang von einer Ionenstärke zur anderen das neue Gleichgewicht wirklich erreicht und nicht in einem falschen Gleichgewicht stecken bleibt. ATP macht offenbar die Fadenmoleküle beweglich, d.h. es setzt auch im Gel ihre Kohäsionskräfte herab. Es scheint somit, als genügte der ATP-Einfluss auf die Kohäsionskräfte des Aktomyosin zur Erklärung der bisher vorliegenden Beobachtungen am Gel wie am Sol. Ob dieser Einfluss allerdings das einzige Prinzip der Wirkung ist, muss solange offen bleiben wie man nicht weiss, warum Pyrophosphat auf Aktomyosinlösungen ähnlich oder gleich wirkt wie ATP, während es Aktomyosinfaden nicht beeinflusst<sup>32</sup>.

Die umgekehrte Wirkung, die Wirkung des Myosins auf das ATP ist einem gewissen, vorläufigen Abschluss der Erkenntnis zugeführt durch die Entdeckungen von Polis

UND MEYERHOF<sup>24</sup>. Die ATPase-Wirkung des Myosin ist zusammen mit einer kleinen Eiweissmenge abtrennbar, ohne dass die Wirkung verloren geht! Das ATPase-Ferment-eiweiss gehört also offenbar nur in soweit zum Myosin, als es auf den Myosinkomplex gebunden ist. Auf Grund der Monodispersität dieses Komplexes—eben des L-Myosin—liegt es allerdings nahe anzunehmen, dass es sich hier nicht um einen zufälligen, sondern um einen stöchiometrischen Komplex handelt.

Und so schliesst heute die Betrachtung der Muskeleiweisskörper mit dem Namen O. Meyerhof, mit dem sie vor einem guten Vierteljahrhundert begann.

#### ZUSAMMENFASSUNG

Im Rahmen eines kurzen zusammenfassenden Berichtes über Muskelproteine werden einige neue Tatsachen gebracht:

- I. Die Diskrepanz zwischen der parallelfaserigen Struktur des I-Abschnittes und dem Fehlen von Stäbchendoppelbrechung der I-Bande kann erklärt werden, wenn man annimmt, die Stäbchen beständen aus dem stark negativ doppelbrechenden N-Protein von Szent-Györgyi. In der I-Bande würden sich dann die positive Stäbchendoppelbrechung dieses Proteins und seine negative Eigendoppelbrechung gerade aufheben.
- 2. Die L-Myosinkomponente und die Aktomyosinkomponenten des Myosin können sauber getrennt werden.
- 3. Es werden für die einzelnen isolierten Komponenten Sedimentationskonstanten, Viskositäten und Werte für die Lichtstreuung angegeben und ebenso die Änderungen dieser Werte bei Zusatz von ATP oder Aktin.
- 4. Eine häufig vorkommende Komponente des Myosin besteht aus einer scharf abgegrenzten Denaturierungsstufe des L-Myosin.
- 5. Aktin und L-Myosin vereinigen sich stufenweise zu Aktomyosinen ganz verschiedener Sedimentationskonstanten.
  - 6. Das L-Myosin sedimentiert und diffundiert monodispers.
  - 7. Das L-Myosinteilchen ist ein Stäbchen von 2200-2400 Å Länge und 22-23 Å Dicke.
- 8. Die beobachteten ATP-Wirkungen können vorläufig sowohl im Sol wie auch im Gel als eine reversible Verminderung der Kohäsionskräfte zwischen L-Myosin und Aktin befriedigend behandelt werden.

#### SUMMARY

The information available on muscle proteins is reviewed and in addition the following new facts are presented:

- I. The discrepancy of the parallel-fibred structure of the I-band and the lack of the form birefringence might be explained by supposing that the micelles consist of the strongly negative birefringent N-Protein of Szent-Györgyi. Thus the positive form birefringence of this protein is compensated by its own negative birefringence.
  - 2. It is possible to separate completely both components: L-myosin and actomyosin.
- 3. The sedimentation constants, viscosities, and values for light scattering of the isolated compounds are given. The changes of these values produced by addition of ATP or actin are also indicated.
- 4. It is shown that one component of the myosin which is frequently found consists of a sharply limited stage of denaturated L-myosin.
- 5. Actin and L-myosin combine step by step to actomyosins of quite different sedimentation constants.
  - 6. The sedimentation and diffusion of L-myosin is monodispers.
  - 7. The L-myosin particle is a micelle with a length of 2200-2400 Å and a diameter of 22-23 Å.
- 8. The observed effects of ATP in sol as well as in gel may satisfactorily be interpreted as a reversible weaking of the cohesive forces linking L-myosin and actin.

#### RÉSUMÉ

Quelques faits nouveaux sont décrits dans un rapport sur les protéines du muscle.

r. En supposant que les micelles soient formées par la N-protéine de Szent-Györgyi à réfraction double négative, il est possible d'interpréter la discordance entre la structure fibrillaire du segment I du muscle et le manque de la réfraction double. Dans ce cas, la positivité de la réfraction double formale pourrait être compensée par la négativité de la réfraction double propre de la même protéine.

- 2. On peut séparer complètement les deux constituants L-myosine et actomyosine.
- 3. Les constantes de sédimentation, les viscosités et les valeurs de l'absorption apparente des constituants isolés sont décrites. En plus, les variations de ces valeurs produites par l'addition d'ATP ou d'actine sont données.
- 4. Il est démontré, qu'un constituant de la myosine fréquemment trouvé est une fraction exactement délimitée de L-myosine dénaturé.
- 5. L-myosine et actine se combinent en plusieurs étapes formant des actomyosines avec des constantes de sédimentation complètement différentes.
  - 6. La sédimentation et la diffusion de L-myosine sont monodisperses.
  - 7. Une particule de L-myosine a une longueur de 2200-2400 Å et un diamètre de 22-23 Å.
- 8. A l'état actuel les effets observés de l'ATP, en solution ou en gel, peuvent être interprétés comme une diminution réversible des forces d'union entre L-myosine et actine.

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### MODIFICATIONS DANS LA

# STRUCTURE PHYSICO-CHIMIQUE DE L'ÉDIFICE CONTRACTILE AU COURS DU CYCLE DE LA CONTRACTION MUSCULAIRE

par

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#### INTRODUCTION

Pendant fort longtemps, les recherches effectuées sur le muscle, et qui ressortissaient de trois disciplines différentes: la morphologie, la physiologie et la biochimie, sont restées sans connections; les techniques auxquelles ces domaines devaient faire appel étaient de nature trop différente et les résultats obtenus par les divers chercheurs offraient peu de recoupements. Nul n'ignore encore le rôle de pionnier que notre Maître O. MEYERHOF, que nous fêtons ici, a joué dans ce rapprochement, si extraordinairement fécond, entre la physiologie et la biochimie du muscle. Ses travaux sont si classiques, si nombreux, constituent un exemple si merveilleux de logique, de profondeur et de perspicacité, qu'ils forment une gerbe modèle dont nous sommes loin d'avoir cueilli aujourd'hui tous les épis. Je ne puis évoquer sans une certaine émotion des notions — comme celles qui établissent les relations quantitatives entre le travail du muscle et son métabolisme — qui nous sont devenues maintenant si familières que nous avons presque oublié qu'elles ne furent pas tout de suite évidentes et qu'il a fallu bien du génie et du talent pour les établir; je ne puis contempler sans émerveillement la liste des enzymes qui interviennent dans le cycle des générateurs d'énergie du muscle et dont un si grand nombre ont été découverts par ce Maître.

Transformations moléculaires d'une part, travail musculaire de l'autre: qu'avonsnous entre les deux?

Que sait-on aujourd'hui du mécanisme grâce auquel l'énergie chimique est transformée en travail mécanique?

Hélas, la route est difficile. Les deux domaines se recoupent au niveau de la machine musculaire, formée de protéines de structure qui sont d'autant plus difficiles à étudier qu'elles existent, in vivo et in situ, non pas comme la plupart des protéines-enzymes: librement dissoutes dans le suc musculaire et par conséquent aisément extractibles sans trop de risques de modifications, mais sous une forme d'association très particulière qui assure précisément cette structure. Les procédés d'extraction nous forcent à briser celle-ci pour ne retrouver, dans nos extraits, que des morceaux dont le degré de dispersion, l'orientation spatiale, la structure et les groupements prosthétiques éventuels sont indiciblement bousculés. Si nous commençons aujourd'hui à connaître un certain nombre de propriétés de ces protéines de structures, considérées in vitro, disons le tout de suite:

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nous sommes fort loin de pouvoir nous représenter l'édifice contractile *en place dans le muscle* et toute tentative consistant à expliquer comment, par l'intervention de cet édifice, les générateurs d'énergie produisent un travail, ne peut être par conséquent que fort spéculative et tout au plus une source plus ou moins suggestive d'hypothèses de travail, ce qui n'est d'ailleurs pas un faible mérite.

Le nombre de protéines de structure qui ont été l'objet de recherches est déjà considérable: citons la myosine (elle-même vraisemblablement complexe: myosines  $\beta_1$ ,  $\beta_2$  (Dubuisson¹),  $\gamma$  (Dubuisson²), l'actine (Straub³, 4, 5) (sous la forme monomère: G-actine et polymère: F-actine), la combinaison des actines aux myosines (F-actomyosine, G-actomyosine6), la tropomyosine de Bailey7, la N protéine de Gerendàs et Matoltsy8. On possède les méthodes pour extraire ces protéines et les séparer les unes des autres et les résultats obtenus sont déjà qualitativement et quantitativement très reproductibles.

Ces techniques ont toutes en commun d'attaquer la pulpe musculaire, préalablement finement divisée par des procédés mécaniques, par des solutions dont les caractéristiques principales ne sont pas tant de posséder une action spécifique sur la solubilité de ces molécules que d'avoir une influence spécifique sur leur extractibilité, c'est-à-dire une action disruptive sur les forces qui maintiennent en place ces protéines de structure.

Tous ceux qui ont extrait ces protéines savent cela et je n'enfonce qu'une porte ouverte en le répétant. Mais peut-être n'a-t-on pas suffisamment songé au parti que l'on peut en tirer, sur un terrain où la physiologie rencontre cette biochimie particulière. Personne ne contestera que l'édifice contractile doit posséder, à l'état raccourci, une structure bien différente de celle qu'il possède à l'état relâché. Cette différence: c'est le noeud du problème. Elle implique un remaniement des éléments constitutifs, des modifications des relations spatiales, physico-chimiques, des changements dans les modes de liaison. On peut ainsi, a priori, prévoir que l'extractibilité des protéines de structure ne peut être la même si l'on part de pulpe de muscle contracté ou de muscle au repos. Et l'on saisit aussi tout de suite que, dans la mesure où il est possible:

- a) de préparer des pulpes musculaires répondant à ces deux états extrêmes du cycle contractile: l'état de *relâchement* et l'état de *contracture*;
- b) d'analyser qualitativement et quantitativement le composition protidique de ces extraits;
- c) d'établir l'existence de changements d'extractibilité de l'une ou l'autre de ces protéines de structure:

l'on se trouve à même d'aborder le problème de la contraction musculaire par un nouvel angle, à la fois physiologique et biochimique et, par conséquent, de nature à apporter des renseignements inédits au problème général de la connaissance du mécanisme de la fonction<sup>9</sup>, <sup>10</sup>.

C'est dans ce domaine que mes collaborateurs et moi travaillons depuis un certain nombre d'années.

Je voudrais ici offrir à mon Maître O. MEYERHOF, sous la forme d'un aperçu général de nos résultats\*, les fruits de notre modeste contribution à l'étude du problème de la contraction musculaire, dont il fut l'un des plus intenses animateurs.

<sup>\*</sup> Les travaux effectués dans notre laboratoire, et dont il sera question dans cet article, sont cités dans les références sous les numéros: 1, 2, 9, 10. 11, 12, 13, 14, 15, 16, 17, 28, 29, 34, 36, 37.

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I. PRÉPARATION D'EXTRAITS PROTIDIQUES DE PULPES DE MUSCLES DE LAPIN SE TROUVANT DANS UN INSTANT DÉFINI DU CYCLE DE LA CONTRACTION

La préparation d'extraits musculaires quelconques nécessite toujours a) la division mécanique du tissu, b) l'extraction à basse température pour éviter les dénaturations, autolyses, etc.

I. Lorsqu'il s'agit de muscles normaux et au repos, il faut que ni le hachage, ni l'abaissement de température n'entraînent une stimulation des fibres musculaires. De nombreux tâtonnements ont montré que le procédé le plus sûr consiste tout d'abord à refroidir le muscle, non pas brutalement en le plongeant dans l'eau glacée ou l'air liquide, ce qui conduit à coup sûr à une certaine stimulation ou même une contracture, au moins des fibres périphériques<sup>11</sup>, mais graduellement, en plaçant les muscles non encore excisés dans une chambre froide (1 à 2° C) pendant au moins une heure. On peut ensuite hacher le tissu au moyen d'un broyeur à viande du genre Latapie, ou placer le muscle refroidi dans une enceinte à -20 à -30° C, dans laquelle le muscle se congélera et pourra être ensuite coupé au microtome à congélation\*, en tranches de 20 à 40  $\mu$  d'épaisseur. Ce dernier procédé fournit des extraits plus riches et de composition plus constante que l'autre<sup>11</sup>.

S'il s'agit d'obtenir des muscles se trouvant à *l'état de raccourcissement maximum*, provoqué, par exemple, par la stimulation électrique, le seul moyen connu d'immobiliser le tissu en cet état consiste à le plonger dans l'air liquide. Nous avons montré que le procédé est moins sûr que l'on pouvait a priori le supposer: le refroidissement brusque paralyse, dans une certaine mesure, les processus d'excitation au niveau de certaines fibres avant que celles-ci aient pu être saisies par la congélation. Aussi observe-t-on fréquemment, au moment de l'immersion — bien que le tétanos électrique soit maintenu —, un relâchement musculaire, plus ou moins considérable<sup>9, 10</sup>. L'obtention, par cette méthode, de fibres musculaires contractées est donc souvent un effet du hasard et nécessite un certain tâtonnement.

Plus sûr à obtenir est l'état de raccourcissement maximum que fournissent certains agents ou facteurs contracturants tels le monoiodoacétate<sup>12</sup>, la strychnine<sup>13</sup>, le *rigor mortis*<sup>13</sup>.

2. Quel que soit le procédé utilisé pour la dilacération du tissu, la mise en solution de certaines protéines demande la présence de solutions salines, dont l'action doit être plus ou moins prolongée et facilitée par une agitation appropriée, suffisamment douce cependant pour éviter l'apparition de mousse dont on connaît l'influence, par action de surface, sur la dénaturation des protéines. Comme on le sait, l'action dissolvante des solutions salines sur les globulines est, grosso modo, proportionnelle à  $\mu = \sum {}^{1} \frac{1}{2}$  CV², où C est la concentration des ions et V leur valence, à condition de rester en deçà de la limite du salting out. Mais la nature des ions n'est pas sans influence; elle dépend de leur degré d'hydratation et de leur pouvoir de s'associer aux protéines. Il en résulte que l'utilisation de solutions de composition diverse conduit à l'obtention d'extraits qui peuvent révéler des richesses dissemblables en protéines. Les différences constatées peuvent porter sur la "qualité" comme sur la "quantité" de protéines extraites. (C'est ainsi que l'actomyosine est plus aisément mise en solution dans les solutions de KCl que dans les solutions de MgCl<sub>2</sub>, de même force ionique, et que (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> extrait peu de myosine  $\beta$ ,

 $<sup>^\</sup>star$  Nous utilisons pour cela un microtome à congélation dont le mouvement est entraı̂né par un moteur électrique, ce qui permet de débiter 100 g de muscle en dix minutes.

qui paraît dénaturée *in situ* sous l'influence de ce sel<sup>9</sup>). Ce qui nous paraît essentiel, c'est d'éviter, autant que possible, *du moins au cours d'une première étape de ce genre d'études*, l'utilisation d'électrolytes dont on sait par avance l'influence nuisible sur le degré de polymérisation de certaines protéines (KI), sur la grandeur des particules (urée), sur leur solubilité (Ca, métaux lourds). Le plus prudent est d'employer des ions ''naturels'' tels que K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, HPO<sub>4</sub><sup>-</sup>-, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, aux p<sub>H</sub> les plus physiologiques possible.

3. Jusqu'à quel point ces méthodes d'extraction permettent l'obtention de solutions de protéines inaltérées est un problème qu'il convient tout d'abord de bien poser. Il est évident que les solutions de protéines obtenues à la suite d'une extraction aussi prudente que possible ne peuvent jamais être considérées comme des solutions au sein desquelles les molécules sont dispersées sous une forme identique à celle qui existe à l'intérieur du myone. Les protéines peuvent être associées, in vivo, de façon bien plus complexe et avoir été dissociées d'un support insoluble, ou séparées de groupements prosthétiques qui y sont naturellement attachés, par l'action même des solutions salines d'extraction. Elles peuvent aussi se trouver, in vivo, sous une forme orientée, peu soluble, passer dans les solutions d'extraction dans un état beaucoup plus dispersé, plus chaotique, et y présenter une structure secondaire (enroulements ou déplissements des chaînes principales) totalement différente. Lorsque nous envisageons des conditions d'extraction qui fournissent un minimum d'altération des constituants protidiques, ceci veut signifier, par conséquent, que ces conditions seront celles dans lesquelles s'observera un minimum de dénaturation, c'est-à-dire de formation de produits insolubles, sans préjuger des autres modifications que nous venons d'envisager. Nous trouvons, en effet, dans le muscle, un exemple curieux fourni par les myosines  $\beta$ . Ces protéines, une fois isolées, sont solubles au  $p_{\rm H}$  7.2 et à  $\mu$  0.20. Cependant une solution d'extraction de cette composition n'extrait que très peu de myosine  $\beta$  d'une pulpe musculaire finement divisée: il faut utiliser des solutions de force ionique comprises entre 0.5 et 1 pour extraire au maximum ces myosines; mais une fois dispersées, on peut garder ces protéines en solution à  $\mu$  0.209. Nous sommes ici en présence d'un cas typique d'extraction d'une molécule qui n'existe sûrement pas, in vivo et in situ, dans l'état où nous la trouvons dans l'extrait; mais, sans autres recoupements, il n'est pas possible de dire si ce fait est dû à une moindre solubilité, in vivo, parce que la molécule présenterait une orientation pseudo-cristalline de ses molécules ou parce qu'elle y serait combinée avec d'autres substances, sous la forme d'un complexe insoluble, mais que les solutions salines dissocient. Nous trouverons encore plus loin d'autres exemples analogues.

Mais c'est là précisément une situation des plus précieuses pouvant contribuer à éclaircir le problème des transformations physiologiques in vivo et in situ, des protéines musculaires. A égalité de conditions d'extraction, si deux muscles, considérés à des états fonctionnels différents, fournissent systématiquement des extraits de composition dissemblable, c'est précisément parce que les forces de liaison sont plus solides dans l'un des deux cas, forces que la solution d'extraction n'est pas capable de briser. Et ceci montre combien il est important, dans la poursuite de ce genre d'études, d'utiliser des solutions dont l'action sur les protéines et les forces de liaison qui les unissent soit aussi tempérée que possible, par la nature et la concentration des ions qu'elles contiennent comme par leur p<sub>H</sub>. On sait, à propos de ce dernier facteur, que le p<sub>H</sub> des solutions d'extraction a une influence considérable sur la stabilité des extraits soumis à la dialyse. Jacob<sup>14</sup>, <sup>15</sup> a montré, dans mon laboratoire, par l'étude systématique d'extraits dialysés

 $_{4}$ 8 heures à toute une série de  $p_{H}$ , la formation de complexes d'agrégation dénaturés dans les zones acides et établi que la zone de sécurité est relativement étroite et se confond avec les  $p_{H}$  biologiques: 6.5-7.6.

# II. TECHNIQUE RENDANT POSSIBLE L'ANALYSE QUANTITATIVE ET QUALITATIVE DES EXTRAITS AVEC UN MINIMUM D'ALTÉRATION

La méthode la meilleure sera évidemment celle qui permettra une analyse des extraits avec un minimum de manipulations: il faut tâcher de ne point modifier le p<sub>H</sub>, la force ionique, la concentration relative de chaque constituant, etc. On sait que deux splendides techniques nous permettent, aujourd'hui, d'analyser des extraits dans de semblables conditions: l'ultracentrifugation et l'électrophorèse. La dernière, due surtout aux recherches de Tiselius, a été, de beaucoup, la plus utilisée; elle est généralement plus facilement accessible aux laboratoires de biochimie et fournit des résultats plus sélectifs que l'ultracentrifugation; nous l'employons intensivement pour l'étude des protéines musculaires depuis 1942. Elle permet de déterminer, par la mesure des déplacements de frontières protidiques (gradients), sous l'influence d'un champ électrique: le nombre de constituants présents, la vitesse de chacun d'eux au p<sub>H</sub> choisi et, par conséquent, le p.i. (vitesse nulle), les proportions de chacun des constituants par la mesure des surfaces occupées par chaque gradient sur les clichés et, dans une certaine mesure, leur degré d'homogénéité, c'est-à-dire la tendance à l'"étalement" de ces gradients dans le temps, qui résulte à la fois de cette hétérogénéité et des phénomènes de diffusion moléculaires. La seule manipulation à faire subir aux extraits consiste à les dialyser pendant au moins 40 heures, à 0° C, contre une solution de p<sub>H</sub> et de force ionique choisie.

Il convient de préciser que les diagrammes électrophorétiques correspondant à des extraits tissulaires ne peuvent révéler toutes les protéines présentes dans cet extrait: on ne peut pratiquement déceler une composante que si sa concentration dans l'extrait dépasse, en valeur absolue, 0.02%, par la méthode de Tiselius-Longsworth<sup>16, 17</sup>. Comme la concentration totale en protéines des extraits dialysés est rarement supérieure à 3%, seuls sont décelables les constituants dont le taux, dans l'extrait, est supérieur à 2%. Encore faut-il que les substances présentes seulement en faibles quantités possèdent, au p<sub>H</sub> considéré, une vitesse qui ne soit pas trop voisine de celle d'autres constituants. Dans le cas du muscle, le nombre important de gradients de protéines et leur hétérogénéité moléculaire font que ces gradients se séparent en général incomplètement; les conditions sont donc défavorables pour mettre en évidence la présence de protéines dont la concentration est peu importante. Or, beaucoup de protéines (surtout les protéines-enzymes) existent dans le muscle à des concentrations faibles; ainsi s'explique le nombre relativement restreint de gradients différents dans les tracés d'électrophorèse, alors que les travaux enzymologiques nous laissent prévoir la présence, dans le muscle, d'un nombre beaucoup plus considérable de protéines solubles dans les solutions salines (une cinquantaine peut-être?).

### III. EXISTENCE DE MODIFICATIONS D'EXTRACTIBILITÉ DE CERTAINES PROTÉINES MUSCULAIRES DU LAPIN SELON LE MOMENT DU CYCLE DE LA CONTRACTION

Aucune description ne peut remplacer l'examen et le commentaire des deux figures ci-dessous qui représentent, chez un même Lapin (muscles homolatéraux), d'une part, Bibliographie p. 36/37.

le cliché électrophorétique d'un muscle normal et au repos, haché et extrait pendant une heure, au moyen de 1.5 volumes de Na<sub>2</sub>HPO<sub>4</sub>: 0.048 m — Na<sub>2</sub>HPO<sub>4</sub>: 0.006 m — NaCl: 0.20 m (24 heures de dialyse contre la même solution) et, d'autre part, celui d'un muscle contracté, immobilisé en cet état dans l'air liquide et traité ensuite de la même façon que le muscle témoin\*.

Ces deux clichés montrent que ces muscles fournissent des extraits différents en plusieurs points.

1. En ce qui concerne le groupe des *myogènes*, nous sommes ici en présence en réalité d'une collection de protéines, de vitesses électrocinétiques fort semblables, qui presque toutes apparaissent déjà dans les extraits *aqueux* de muscles et qui doivent être considérées comme des protéines existant, *in vivo et in situ*, sous une forme soluble<sup>9</sup>. Ces pro-

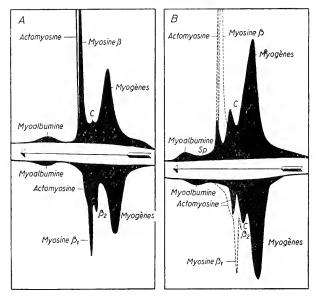


Fig. 1. Protéinogrammes électrophorétiques (méthode de Tiselius-Longsworth) d'extraits musculaires du Lapin,  $\mu$ : 0.35, pH: 7.40,  $\sim$  50000 secondes d'électrophorèse. En A, muscles normaux et au repos, refroidis lentement. En B, muscles contractés par stimulation et immobilisation dans cet état par congélation instantanée. En traits interrompus: les gradients de l'actomyosine et des myosines  $\beta_1$  et  $\beta_2$  du muscle normal au repos.

téines comprennent le myogène de Weber<sup>18</sup>, les myogènes A et B de Baranowski<sup>19–22</sup>, l'aldolase<sup>22b</sup> (qui est une partie du myogène A de Baranowski (Engelhardt<sup>23</sup>, Meyerhof et Beck<sup>24</sup>), la glycéraldéhyde déshydrogénase (Cori, Stein et Cori<sup>25</sup>), la phosphoglucomutase (Najjar<sup>26</sup>) et probablement bien d'autres protéines-enzymes dont la vitesse électrocinétique ne nous est pas encore connue<sup>9, 17, 27</sup>.

Rien ne permet de distinguer le groupe d'ensemble de ces myogènes dans les extraits de muscles au repos ou de muscles contractés; il semble bien que la distribution quantitative et qualitative de ces protéines dans les extraits ne subisse pas de modification au cours du cycle de la contraction<sup>14, 28, 29</sup>.

<sup>\*</sup> Nous n'envisagerons pas, dans cet article, le cas du muscle épuisé par stimulations répétées dont le cas s'apparente plus à l'étude du métabolisme musculaire qu'à celle du mode de fonctionnement de l'édifice contractile (Dubuisson<sup>27</sup>).

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- 2. Aucune différence ne s'observe non plus au niveau du gradient h (Jacob<sup>14</sup>) qui représente la myoalbumine de Bate-Smith<sup>30</sup>.
- 3. En dehors du cas des myogènes et de la myoalbumine, la distribution de tous les autres constituants est modifiée dans l'état de contraction.

Ces autres constituants sont:

a. les myosines. Electrophorétiquement, la myosine classique de Weber-Edsall $^{31,32}$  préparée selon Greenstein et Edsall $^{33}$ , à partir de muscles au repos, est caractérisée par trois gradients que nous avions appelé à l'époque de ces recherches: myosines a,  $\beta$  et  $\gamma^2$ ,  $^{34,9}$ . Banga et Szent-Györgyi $^{35}$  ont montré que ces préparations de myosine, selon Greenstein et Edsall, contiennent deux constituants: la myosine proprement dite et une combinaison de cette myosine (actomyosine) à une protéine du stroma, l'actine, plus tard isolée par Straub $^{3-5}$ , et que l'on peut obtenir des échantillons contenant des taux variables de ces deux constituants en faisant varier le temps d'extraction: plus celui-ci est prolongé, plus il y a de l'actomyosine en solution.

Ayant réussi plus tard à séparer deux myosines,  $\alpha$  et  $\beta^{36}$ , des trois constituants électrophorétiques de la myosine, nous avons pu montrer que le gradient  $\alpha$  correspond réellement à l'actomyosine de Szent-Györgyi et le gradient  $\beta$  à la myosine proprement dite. La myosine  $\gamma$ , d'ailleurs très faiblement représentée dans ces extraits, n'a pas encore pu être isolée.

L'aspect des gradients actomyosine et myosine des extraits totaux de muscles normaux est caractéristique<sup>2, 34</sup>. Ces deux gradients, de vitesse voisine, ne se séparent que lorsque les électrophorèses sont suffisamment prolongées. Le premier (actomyosine) est toujours beaucoup plus aigu que le gradient de la myosine dans le compartiment ascendant de la cellule d'électrophorèse. La forte viscosité des solutions d'actomyosine freine considérablement les phénomènes de diffusion qui sont la cause principale de l'étalement des gradients; en outre, le gradient d'actomyosine sépare nettement la colonne de protéines en deux régions: l'une turbide et une autre non turbide (les solutions d'actomyosine possèdent une turbidité élevée).

Dans le compartiment descendant, le gradient actomyosine est, au contraire, fortement étalé dans les extraits totaux. Cet aspect dissymétrique existe aussi pour le gradient de la myosine qui paraît unique du côté ascendant, mais nettement bifide du côté descendant ( $\beta_1$  et  $\beta_2$ ). Les raisons de ces asymétries sont encore peu évidentes; elles résultent sans doute d'interactions entre l'actomyosine et la myosine, car si l'on étudie électrophorétiquement des solutions pures d'actomyosine ou de myosine, les figures ascendantes et descendantes sont symétriques pour chacune de ces protéines<sup>36</sup> (compte tenu de la dissymétrie classique due au principe même de la méthode électrophorétique).

Les caractéristiques électrocinétiques (en 10<sup>-5</sup> cm/volt/sec) de ces deux gradients sont (μ: 0.40 (Na<sub>2</sub>HPO<sub>4</sub>: 0.048 m — NaH<sub>2</sub>PO<sub>4</sub>: 0.006 m — NaCl: 0.25 m, p<sub>H</sub>: 7.3 à 7.4<sup>9</sup>):

Si l'on se reporte maintenant aux extraits de muscles contractés, les différences sont extrêmement grandes. Il n'y a plus ici qu'une très faible quantité d'actomyosine visible cette fois, à l'anode comme à la cathode, tandis que le gradient des myosines  $\beta$  Bibliographie p. 36/37.

a complètement disparu<sup>1</sup>. (Ce qui explique précisément la visibilité des gradients d'actomyosine et à l'anode et à la cathode).

Mais il existe encore d'autres différences entre le muscle normal et le muscle contracté. Dans ce dernier cas, les extraits contiennent une composante nouvelle, que nous avons appelé provisoirement "contractine" et qui est toujours absente ou faiblement représentée dans les extraits de muscles normaux (dont les fibres ne sont d'ailleurs pas toujours exemptes d'un certain degré de contracture<sup>10</sup>). Les caractéristiques électrocinétiques de cette nouvelle composante sont, dans les mêmes conditions que celles mentionnées ci-dessus<sup>9</sup>:

Enfin, dans les extraits de muscles contractés, entre les gradients formés par l'actomyosine et la myoalbumine, on voit accumulé une certaine quantité de matériel protidique très hétérogène, sp, visible aussi bien du côté descendant que du côté ascendant et qui représente une augmentation notable du matériel sp toujours présent, mais en faibles quantités, dans les extraits de muscles normaux.

Ajoutons que les constatations décrites ci-dessus sont valables, quelles que soient les causes de la contracture (ac. monoiodoacétique, strychnine, rigor mortis) et identiques aux cas de contraction par stimulation et immobilisation par l'air liquide<sup>13</sup>.

#### DISCUSSION

Inextractibilité totale des myosines  $\beta$  par KCl, apparition de contractine et de certaines protéines du groupe sp, voilà des faits essentiels qui caractérisent la contraction ou la contracture, quelle que soit la cause de celle-ci. Et le parallélisme entre le degré de raccourcissement et ces modifications protidiques est si étroit qu'en cas de contracture incomplète (rigor mortis en voie de formation), on peut observer des états intermédiaires caractéristiques<sup>13</sup>.

Or, si au lieu par exemple d'immobiliser le muscle, amené en contraction par un bref tétanos, dans l'air liquide, on interrompt l'excitation pour le laisser se relâcher, il fournira le même extrait protidique que le muscle normal. Les modifications d'extractibilité du muscle contracté doivent donc être réversibles; elles ne se constatent que si l'on saisit la machine "sur le vif".

Examinons tout d'abord le cas des myosines  $\beta$ . Le passage en solution de ces myosines ne peut être une simple dissolution. Tout d'abord, les quantités de cette substance que l'on peut extraire d'un muscle dépendent du degré de division du tissu, ce qui n'est point le cas pour les protéines appartenant au groupe des myogènes<sup>11</sup>. Les muscles, finement divisés au moulin à viande genre Latapie fournissent — toutes autres conditions étant égales — moins de myosine que les muscles coupés finement au microtome à congélation en tranches de 0.02 mm<sup>11</sup>. Les myosines appartiennent donc à des structures spatialement peu accessibles aux solutions salines, sans doute parce qu'elles sont protégées par des structures morphologiques. Rappelons ensuite (voir p. 28) que de nombreux dosages nous ont montré que l'extraction des myosines  $\beta$  nécessite des solutions plus concentrées que celles qui permettent de garder simplement en solution ces mêmes myosines. Ces substances isolées sont en effet très solubles à une force ionique de 0.20 à 0.25 (KCl 0.25 m, de  $p_{11}$  7.00); mais si l'on fait agir semblable solution sur la Bibliographie p. 36/37.

pulpe musculaire, on extrait seulement  $^1/_5$  des myosines que l'on peut obtenir si l'on traite la pulpe musculaire avec une solution de KCl o.6 m³. Ceci indique qu'entre les myosines  $\beta$  isolées et les myosines  $\beta$  telles qu'elles existent in situ dans le muscle, il existe de profondes dissemblances que l'on peut sans doute rapporter au fait que, dans ce dernier cas, ces myosines font partie de structures complexes dont elles se dissocient d'autant plus aisément qu'on les attaque par des solutions salines concentrées.

A la lumière des travaux de l'école de SZENT-GYÖRGYI<sup>6, 36</sup>, qui montrent l'affinité de la myosine pour cette protéine du stroma: l'actine, on pourrait penser que les structures complexes auxquelles nous venons de faire allusion sont constituées par de l'actomyosine. Mais si tel était le cas, il faudrait admettre que la solution d'extraction brise les forces de liaison entre l'actine et les myosines  $\beta$  (ces forces paraissent devoir être des ponts SH<sup>37, 38</sup>) et permette la dispersion de cette dernière dans l'extrait, tandis que l'actine resterait insoluble dans les conditions de nos extractions. Malheureusement, les solutions d'actomyosine ne sont jamais scindées en actine et en myosine sous l'influence de sels (KCl: 0.6 m); s'il en était autrement, il ne pourrait jamais y avoir d'actomyosine dissoute dans une solution saline.

Quoi qu'il en soit de la nature du complexe auquel sont normalement associées les myosines  $\beta$ , nous devons admettre, puisque ces myosines sont devenues inextractibles dans la pulpe de muscles contractés ou contracturés, que le raccourcissement a modifié leurs forces de liaison: elles sont désormais inaccessibles aux solutions salines utilisées. Il est sans doute assez pertinent de penser que c'est l'établissement de ces forces de liaison même qui entraîne la mise en tension (contraction isométrique) ou le raccourcissement (contraction isotonique) de la machine contractile et leur disparition qui assure son relâchement.

En ce qui concerne la contractine<sup>1</sup>, on peut envisager plusieurs causes à son apparition dans les extraits de muscles contractés. Il est tout d'abord possible que la contractine, dont l'apparition accompagne la disparition des myosines  $\beta$ , soit en réalité une partie des protéines  $\beta$  transformée, par exemple, par le gain ou la perte de quelque groupement prosthétique qui en modifierait les propriétés électrocinétiques. Signalons cependant qu'il ne semble exister aucune relation quantitative entre la disparition des myosines  $\beta$  et l'apparition de contractine dans le cas des contractures non maximales. Il est possible d'admettre aussi que l'on a affaire à une protéine qui devient extractible lorsque la machine est à l'état raccourci, parce qu'elle est libérée à ce moment de complexes, ordinairement indissociables par les solutions salines. On en arriverait en somme, dans cette dernière éventualité, à constater, pour la contractine, l'inverse de ce qui se présente pour les constituants de la myosine  $\beta$ , qui ne sont plus libérables par KCl, lorsque le muscle est à l'état contracté<sup>9</sup>.

Quant à la nature de la contractine, nous savons seulement ceci: cette protéine précipite mal dans les conditions où précipite le myosine de Weber-Edsall (actomyosine + myosines  $\beta$ ,  $\gamma$ ), soit à  $\mu$ : 0.05 et au p<sub>H</sub> 6.3. Elle ne peut être extraite du muscle contracté à une force ionique inférieure à 0.15–0.20. Elle ne peut non plus correspondre à la phosphorylase b de Cori<sup>39</sup>, <sup>40</sup>, qui apparaît dans les muscles fatigués par suite de la transformation de phosphorylase a, car elle n'est jamais présente dans les muscles fatigués par stimulations et relâchés; de plus, le taux de contractine est bien supérieur à celui des phosphorylases. On ne peut exclure, a priori, cette possibilité que la contractine corresponde à cette protéine dont nous avons trouvé des traces dans la plupart des préparations de myosine de Weber-Edsall du Lapin et que nous avons, à cette époque,

appelée myosine γ². Nous l'avions trouvée beaucoup plus abondante dans les préparations de myosine faites à partir de muscles de Mollusques (muscles pédieux), qui sont d'ailleurs des muscles très excitables et qu'on ne peut réduire en pulpe sans en provoquer la contracture. Au p<sub>H</sub> 7.3 à 7.4 et μ 0.35 à 0.40, la vitesse de la contractine est de — 2.35 (asc.) et de — 2.05 (desc.); celle de la myosine γ est, dans les mêmes conditions, pratiquement la même, peut-être un peu plus faible (—2.25) (asc.). Il y a lieu cependant de noter que, contrairement à la myosine γ, la contractine ne précipite pas dans les conditions où précipite la myosine de Weber-Edsall dans laquelle on reconnaît la présence de myosine γ, bien qu'en faibles quantités. Enfin, de récentes analyses électrophorétiques effectuées sur des échantillons de G-actine, de F-actine et de tropomyosine\* montrent que la contractine ne peut être aucune de ces protéines-là. Par contre, il semble qu'existe certaines analogies, qui font l'objet de recherches actuelles, entre la contractine et la N-protéine de Gerendàs et Matoltsy<sup>8</sup> qui entre dans la constitution des portions isotropes des myofibrilles. L'emplacement même de ce nucléoprotéide dans la fibre musculaire donnerait un intérêt particulier à ce rapprochement.

On peut se demander maintenant s'il n'est pas possible de trouver des solutions d'extraction qui possèdent la propriété a) ou bien de briser les forces de liaison qui maintiennent si solidement les myosines  $\beta$  à d'autres substances au moment de la contraction et qui seraient en conséquence susceptibles d'extraire ces protéines d'un muscle contracté ou contracturé; b) ou bien de briser les forces de liaison qui rendent inextractible la contractine des muscles normaux. C'est également ce qui fait l'objet de nos recherches actuelles, dont les résultats préliminaires, fort encourageants, seront publiés sous peu et semblent devoir être de nature à éclairer grandement la connaissance de la structure de l'édifice contractile.

#### CONCLUSIONS

Seules les protéines extraites par les solutions de force ionique élevée doivent être considérées comme des constituants engagés in vivo et in situ, dans des complexes qui sont par eux-mêmes insolubles. Or, il se trouve précisément que ce sont ces protéines là dont l'extraction est la plus modifiée au cours du cycle de la contraction. Il est ainsi tout naturel de penser que le fonctionnement de la machine contractile est essentiellement caractérisé par la formation ou la dissociation de ces complexes. Cette conclusion est en harmonie avec les théories suggérées par Szent-Györgyi<sup>6, 36</sup>, selon lesquelles le mécanisme de la contraction résulterait de la transformation de l'actomyosine sous l'influence de sels et d'A.T.P.; mais ceci n'est qu'une solution approchée, comme le reconnaît d'ailleurs lui-même ce chercheur. Tout d'abord, la myosine elle-même est une substance compliquée. Elle est constituée d'au moins deux composantes électrophorétiques:  $\beta_1$  et  $\beta_2$ ; elle contient l'A.T.Pase<sup>41</sup>, qui est un enzyme n'étant vraisemblablement qu'aceroché à la myosine; elle contient encore d'autres enzymes: une désaminase42, Lapoferment d'un enzyme susceptible de transformer l'arginine et l'histidine en créatine<sup>43</sup>. Le cycle de la contraction affecte aussi une autre myosine: la composante  $\gamma$ , électrocinétiquement distincte de  $\beta^2$ . Le substrat auquel les myosines peuvent se lier contient sûrement l'actine de Straub (sous la forme de F-actine vraisemblablement, étant donnée la force ionique du musele) et peut-être même la tropomyosine de Bailey

<sup>\*</sup> Recherches inédites.

et la nucléoprotéine de Gerendàs et Matoltsy. Enfin, la liaison des myosines à l'édifice contractile au moment de la contraction est concomitante de la libération de la contractine, nettement distincte de l'actomyosine et des myosines  $\beta_1$  et  $\beta_2$ . Ce sont là des faits qui permettent de penser que la machine contractile est beaucoup plus compliquée que l'on serait tenté de le croire. Déjà, les travaux de l'école de Szent-Györgyi ont montré par la découverte de l'actomyosine, que les myofibrilles ne sont pas uniquement constituées de myosine, comme on l'avait cru jusqu'àlors; mais il serait dangereux de penser que le schéma de la contraction musculaire construit sur la base actomyosine — ATP — KCl — MgCl<sub>2</sub> est satisfaisant, malgré ce que certaines expériences faites avec des fils préparés au moyen de cette substance peuvent avoir de spectaculaire (superprécipitation ou forte déshydratation (cynaerèse) sous l'influence de sels ou d'A.T.P.<sup>44–49</sup>). On ne fera certes jamais trop d'expériences dans le genre de celles qui furent faites par NEEDHAM et collaborateurs<sup>50, 51</sup>, ainsi que par l'école de Szent-Györgyi, sur les propriétés des myosines sous l'action de telle ou telle substance; mais on n'en fera jamais assez pour poser tout d'abord, dans toute son ampleur, le problème "physiologique" qui consiste à déterminer combien de protéines appartiennent réellement aux structures dont les modifications assurent le mécanisme de la contraction et du relâchement musculaires et comment se modifient leurs modes de liaison au cours du cycle de la contraction. C'est une première contribution à ce genre d'investigation dont les résultats ont été résumés ici. Ils montrent qu'en s'efforçant de dissocier les complexes protidiques, avec le moins de brutalité possible, en attaquant leurs forces de liaison par des solutions d'extraction de composition appropriée, afin de libérer progressivement les éléments détachables, on peut, par des comparaisons faites sur des muscles se trouvant en divers états fonctionnels (relâchés, contractés) se rendre compte par la méthode électrophorétique, de l'établissement ou de la rupture de liaisons qui unissent les éléments qui participent à la contraction. Les résultats obtenus jusqu'ici sont encore fort difficiles à interpréter et ne peuvent pas encore, pas plus d'ailleurs que ceux obtenus par d'autres voies, servir à construire une théorie de la contraction et du relâchement musculaires. Si certains éléments permettent de penser que le cycle de la contraction est dû à la formation et à la dissociation de complexes constitués d'actine, de myosines  $\beta_1$  et  $\beta_2$ , de contractine, etc., il nous faut encore mieux connaître la structure de ces complexes et leurs modifications au cours du cycle de la contraction. Et ceci est un chemin dont le parcours est encore long et difficile.

#### RÉSUMÉ

L'édifice contractile doit posséder, à l'état raccourci, une structure bien différente de celle qu'il possède à l'état relâché. Cet édifice étant essentiellement constitué de protéines, on doit s'attendre à ce que l'extractibilité de ces substances, au moyen de solutions salines ayant une action plus ou moins disruptive sur les forces de liaison qui maintiennent les protéines en place dans l'édifice, doit être différente selon que l'on considère le muscle à l'état contracté — ou contracturé — ou relâché.

être différente selon que l'on considère le muscle à l'état contracté — ou contracturé — ou relâché. C'est effectivement ce que nous avons pu constater. Pour ne citer que les faits les plus saillants: tandis que les myosines β deviennent inextractibles par les solutions salines utilisées, lorsque la machine musculaire se trouve à l'état contracté, une nouvelle protéine: la contractine apparaît dans les extraits. Ces observations sont discutées. Il apparaît que la méthode d'investigation employée, qui fait appel simultanément à des techniques physiologiques, physico-chimiques et biochimiques, est loin d'avoir fourni tous les renseignements qu'elle est susceptible de nous apporter dans la connaissance du problème du mécanisme général de la contraction musculaire.

#### SUMMARY

The contractile apparatus must possess, in the shortened state, a structure which differs from that in the relaxed state. As it is essentially composed of proteins, one must expect the extractability *Bibliographie p. 36/37.* 

of these substances — as effected by salt solution, possessing a more or less disruptive action on the forces which keep the proteins in their place in the structure — to differ when the muscle is in state of contraction or relaxation.

This we have been able to observe. The most remarkable facts are: When the muscle is in state of contraction the myosins  $\beta$  cannot longer be extracted by the salt solutions employed, but then a new protein, the contractine, appears in the extracts. These observations are discussed. The method of investigation employed, requiring at one time physiological, physico-chemical and biochemical techniques, does not yet appear to have revealed all information it is expected to yield in contribution to the understanding of the mechanism of muscle contraction.

#### ZUSAMMENFASSUNG

Der Kontraktionsapparat muss im verkürzten Zustand eine andere Struktur haben, als im Ruhezustand. Da er grösstenteils aus Proteinen besteht, so ist zu erwarten, dass die Extrahierbarkeit dieser Substanzen mit Salzlösungen aus kontrahiertem und ruhendem Muskel verschieden sein wird, denn die Salzlösungen wirken mehr oder weniger spaltend auf die Bindungen welche die Proteine in der Struktur zusammenhalten.

Wir konnten dies in der Tat beobachten. Nennen wir nur die hervorragendsten Fälle: Wenn der Muskel kontrahiert ist, können die  $\beta$ -Myosine nicht mehr durch Salzlösungen extrahiert werden, aber ein neuer Eiweisstoff, das Kontraktin, tritt in den Extrakten auf. Die verwendete Methode, die gleichzeitig von physiologischen, physiokochemischen und biochemischen Arbeitsweisen Gebrauch macht, scheint noch lange nicht alle Aufklärungen zum Verständnis des Mechanismus der Muskelkontraktion gegeben zu haben, die sie verschaffen könnte.

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# ACTOMYOSIN AND MUSCULAR CONTRACTION

by

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It has been shown in the author's laboratory<sup>10</sup> that two structural proteins can be extracted from the muscle fibril, actin (F. B. STRAUB) and myosin. The two, if mixed at a proper ionic concentration, unite to a complex, actomyosin, which has the remarkable property of contractility. Actomyosin threads contract under influence of ATP. This contraction, though imitating in many ways contraction of muscle, differs from it also in several respects. Two of these differences are rather striking and led Buchthal, DEUTSCH, KNAPPEIS, and PETERSEN, as well as ASTBURY, PERRY, REED, and SPARK to the conclusion that "contraction" of actomyosin has little to do with muscular contraction. According to ASTBURY, "contraction" of actomyosin is simply a colloidal synaeresis while muscular contraction is an entirely different phenomenon. The two observations, on which this conclusion was based, were the following: muscle contracts anisodiametrically, becoming shorter and thicker without changing volume, while "contracting" actomyosin threads become shorter and proportionately thinner, thus simply shrinking. The second objection is based on Buchthal's observation: while an unloaded actomyosin thread shortens in ATP, a loaded thread lengthens in the same solution, thus behaving contrary to muscle which shortens whether loaded or unloaded.

In this paper, the author, after pointing out certain analogies between the contraction of muscle and actomyosin, hopes to show that the objections raised by Buchthal and Astbury can easily be explained and do not plead for a basic dissimilarity of the two processes.

If a washed fibre bundle of the *musculus psoas* of the rabbit is suspended in a Ringer solution, containing o.oor M Mg and 0.2% ATP, it contracts and develops the same tension as the muscle developed maximally *in vivo*, showing that it was the normal mechanism of contraction which has been put into motion by ATP. This reaction is very specific, and all attempts to produce it with any other substance than ATP have hitherto failed. The same muscle fibre can be made to shorten also by other means, as for instance, by heat. At 70° shortening may be extensive, but no appreciable tension will be produced.

If the same washed psoas muscle is suspended in water and decomposed in the Waring blender into a suspension, on addition of the salts of the Ringer solution, a moderate flocculation will be observed. On addition of ATP an excessive precipitation occurs which has been termed "superprecipitation". Evidently, this superprecipitation

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is, in its essence, identical with muscular contraction, having been elicited by the same specific substance under a similar condition, the only difference being the destruction of the fibrillary architecture.

We can go one step further and dissolve out of the freshly minced psoas the contractile matter, actomyosin, by prolonged extraction by means of Weber's alkaline o.6 M KCl. This actomyosin behaves like the suspended muscle giving flocculation in presence of salts and superprecipitation in presence of ATP. The last step of degradation of the muscle may be the isolated extraction of actin and myosin. The two proteins, if mixed, unite to actomyosin which gives the same reactions as actomyosin extracted or the suspended psoas. This stepwise decomposition of the psoas thus gives identical results all the way, and the reactions, elicited by the highly specific ATP, are, in all phases, so similar that there can be little doubt about the essential identity of these reactions. Naturally, we must bear in mind that the fibril has its specific architecture which is present no more in suspensions.

Instead of making a suspension out of our actomyosin, we can also bring it into the form of a gel and make of this gel, by the method of Weber, a fibre again. Suspended in pure water, the thread will swell. Addition of salts will make this swelling regress, a reaction which evidently corresponds to the flocculation of our actomyosin or muscle suspensions. On addition of ATP the thread, if thin enough, will shorten rapidly, a reaction which evidently corresponds to the superprecipitation of our suspensions and corresponds thus, also, in its essence, to contraction in muscle.

After having pointed out these analogies of actomyosin and muscle, let us consider the dissimilarities, quoted above.

Muscle shortens; actomyosin shrinks. This is certainly true, and our problem is whether this difference is due to a difference in the very essence of the reaction or whether it is due merely to the rough structural difference between fibril and actomyosin thread. In the former, as shown by the electron microscopic studies of Hall, Jacus, and Schmitt<sup>8</sup>, the contractile filaments run all along the muscle fibril continuously, parallel to the axis. On extraction these filaments are broken up into fragments which are distributed at random in the actomyosin thread. If, in contracting muscle the filaments become shorter and wider, the muscle will have to do the same — become shorter and wider without changing volume. If the same shortening of filaments occur in the actomyosin thread which contains the fragments unoriented, at random distribution, the shortening of the very same filaments has to make the thread contract equally in all directions, that is make it shrink.

That this difference is actually due only to this difference in orientation can easily be shown. If the thread is gently stretched, as shown by Gerendas, the filaments become oriented parallel to the axis similarly to muscle. If ATP is made to act on such an oriented thread, this thread will shorten and become wider, thus contract without changing volume, similarly to muscle. The same is true, as shown by Buchthal and his associates after drying which acts as stretching.

Perry, Reed, Astbury, and Spark explain the "synaeresis" of actomyosin by a lateral association of particles. That this explanation cannot be correct is shown by the anisodiameteral contraction of the oriented actomyosin threads. In this structure the filaments are oriented parallel to the axis. Their lateral association could only make the thread thinner and never shorter, while the experiment shows that actually the opposite happens and the thread becomes shorter and wider.

In order to be able to discuss the stretching of the loaded actomyosin thread in ATP, we have to give our attention for an instant to another effect of ATP, independent of contraction. Fresh muscle is elastic. Post mortem the ATP is disintegrated and, parallel to its disappearance, the muscle becomes inelastic, as shown by Th. Erdős, Bate-Smith, and Bendall. It is possible to show that it was actually the disappearance of ATP which induced this difference. A washed psoas fibre is inelastic. If suspended in Ringer, containing ATP, it becomes elastic again. This shows that in absence of ATP, links are developed between neighbouring micells which make the system rigid, making slipping and relative motion impossible. These are abolished by ATP. This effect of ATP is independent of its second effect, contraction. If ATP did not have the first effect, it could not induce contraction at all because the system would be too rigid. This effect of ATP was, in fact, the very first specific effect discovered of ATP on "myosin" by Engelhardt, Ljubimowa, and Meitina who found that ATP makes "myosin" threads more extensible. The decrease of dynamic softness of actomyosin induced by ATP has also been studied extensively by Buchthal and his associates.

After this short discussion we can consider now the extension of loaded actomyosin threads. If an actomyosin thread is loaded, it will not stretch because it is rigid, its particles being held together by the links or cohesive forces described before. If ATP is added these forces will be abolished and, under action of the load, the short fragments of filaments of which the thread is composed, will begin to slip under influence of the load, and the thread will lengthen, even if at the same time these fragments shorten. The situation will be different in an unloaded thread. There will be no force present to cause slipping, and the shortening micells will make the thread contract or "shrink" according to its co-axial or random distribution. In the muscle fibre there can be no slipping because the filaments run continuously through the fibrils, and so the muscle can shorten only if its filaments contract, whether loaded or unloaded.

Perry, Reed, Astbury, and Spark stress one more difference between muscular contraction and the contraction in actomyosin threads: the time factor. Muscle may contract several hundred times per second, while even thin threads need seconds for their contraction. Here again the difference lies in steric relations and not in principle. If diffusion and friction are eliminated, the ATP contraction is instantaneous. This can be shown in washed psoas-fibres suspended o° C in a solution. At this temperature the fibres develop only a very weak tension. If they are transferred into a Ringer of, say 25° C, the development of a high tension is instantaneous. Rapid reaction can also be demonstrated in thin actomyosin threads, to which ATP is added in such a way as to reach the thread from one side. On this side the actomyosin contracts and makes the thread bend or curl up rapidly.

The differences in behaviour of muscle and actomyosin can thus, in the instances discussed, be explained satisfactorily by the rough structural differences of both formations and need not be ascribed to the difference in underlying reactions.

#### SUMMARY

It is shown that the contraction of muscle, superprecipitation of its suspensions, superprecipitation of actomyosin and contraction of actomyosin, elicited by ATP, are related phenomena.

Differences in behaviour, as for instance anisodiametry of shrinking in muscle and isodiametry of shrinking in unoriented actomyosin gels, can be explained by the differences in structure. The same is true for the difference of muscle and loaded actomyosin threads, the latter of which, contrary to muscle, lengthen under influence of ATP.

#### RÉSUMÉ

Il a été montré que la contraction du muscle, la superprécipitation de ses suspensions, la superprécipitation de l'actomyosine et la contraction de l'actomyosine, provoquées par l'ATP, sont des phénomènes connexes.

Des différences de comportement, comme par exemple l'anisodiamétrie de rétrécissement du muscle et l'isodiamétrie de rétrécissement de gels d'actomyosine non orientée, peuvent être expliquées par les différences de structure. Le cas est le même en ce qui concerne la différence entre les fibres musculaires et les filaments d'actomyosine chargés, ces derniers s'allongeant, contrairement au muscle, sous l'influence de l'ATP.

#### ZUSAMMENFASSUNG

Es wird gezeigt, dass die Zusammenziehung des Muskels, die Super-Fällung seiner Suspensionen, die Super-Fällung von Aktomyosin und die Zusammenziehung von Aktomyosin, hervorgerufen durch ATP, mit einander zusammenhängende Erscheinungen sind.

Verschiedenheiten des Verhaltens, wie z.B. die Anisodiametrie des schrumpfenden Muskels und die Isodiametrie der Schrumpfung in unorientierten Aktomyosin-Gelen, können durch die Strukturverschiedenheiten erklärt werden. Dasselbe gilt für die Unterschiede zwischen Muskel und belasteten Aktomyosin-Fäden, welch letztere sich im Gegensatz zum Muskel unter der Einwirkung von ATP

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It is a great pleasure and privilege to offer these lines to one of the most distinguished pioneers of muscle research; I wish him long years of undisturbed scientific activity.

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# MYOSIN AND ADENOSINETRIPHOSPHATE IN RELATION TO MUSCLE CONTRACTION

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The conception of energy provision by the splitting off of the terminal phosphate group of ATP, under the influence of myosin or actomyosin acting as ATPase, is central in current hypotheses of muscle contraction. Indeed, in many aspects of metabolism we find evidence that ATP serves as a readily expended store of energy and that much of the free energy of oxidation and glycolysis goes to its resynthesis. In these circumstances, it is strange to reflect that we are still without accurate knowledge of the amount of free energy available in this reaction; we do know, however, that it is surprisingly small, only of the order of 12000 g cals per g/mol H<sub>3</sub>PO<sub>4</sub> set free. Still more surprising is the small difference in free energy content (only about 6000-8000 g cals) which separates the "energy-rich" phosphate bonds from the "energy-poor" phosphate bonds. It is probably because of its ability to deal in these small stages of energy transfer that the living cell achieves its high efficiency. Thus even normal aerobic contraction is about 20% efficient when tension production or work performance is compared with heat production; and anaerobic contraction about 40% efficient. The anaerobic recovery phase (when creatinephosphate formation is going on at the expense of carbohydrate breakdown to lactic acid) is over 90% efficient: there is little heat production during this period and the formation of the energy-rich phosphate bonds goes on with scarcely any waste in the form of heat. We shall return to this point later.

The fact that no breakdown of ATP has been demonstrated in normal contraction, but only becomes observable in fargoing fatigue, has recently been emphasized by A. V. Hill. By the use of the new micro-methods, for example those of Kalckar², it should now be possible to estimate ADP in amounts of the order to be expected during a single twitch or a very short series of twitches. Although rephosphorylation by means of creatine phosphate probably follows with great rapidity, by using slow-moving muscle at low temperature it might thus be possible to detect a period of ATP breakdown unobscured or only partly obscured by resynthesis.

#### ATPASE ACTIVITY in vivo AND in vitro

The close connection of ATP breakdown with energy provision for contraction once conceded, two very important questions arise — the exact conditions of the ATPase activity and its timing.

That myosin can act as ATPase is wellknown<sup>3</sup> but, as Bailey has shown<sup>4</sup>, the optimal conditions for the activity of myosin prepared in the classical manner and containing little actomyosin, are not those to be expected within the muscle fibre. The

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activity is very low around p<sub>H</sub> 7.4, the activity of fresh preparations increasing progressively up to and beyond p<sub>H</sub> 10; Ca<sup>++</sup> is an essential activator and Mg<sup>++</sup> exercises a strong antagonism to Ca<sup>++5</sup>. These facts have led Mommaerts and Seraidarian<sup>6</sup>, to repudiate the possibility that ATP can break down in the fibre at more than a small fraction of the rate required to produce the increase in free phosphate observed on contraction. But here some recent experiments of Keilley and Meyerhof' seem likely to throw important light on a dark place. In a study of the ATPase activity of various protein fractions from muscle, they found with myosin alone the high p<sub>H</sub> optimum and the Ca++-Mg++ antagonism already mentioned; but with actomyosin (made from "'crystalline myosin" and purified actin) they observed in presence of Ca++ an optimum activity around p<sub>H</sub> 7.7, almost unaltered by addition of Mg<sup>++</sup>. Szent-Györgyi<sup>8</sup> had already remarked on Mg++ activation of the ATPase activity of "impure natural actomyosin" but this effect may have been due to presence of myokinase. Mommaerts AND SERAIDARIAN<sup>6</sup> report experiments on ATPase activity of actomyosin at p<sub>H</sub> 7.0 and p<sub>H</sub> 9.0 where Mg<sup>++</sup> showed its antagonistic effect to Ca<sup>++</sup>. It certainly seems that further enzymic examination of actin, myosin and their combinations might lead to illuminating results.

Keilley and Meyerhof<sup>7</sup> describe also the preparation from muscle of a second Mg<sup>++</sup>-activated ATPase, p<sub>H</sub> optimum 6.8, containing no myosin or actin, but possibly associated with mitochondrial particles; this may correspond to the ATPase found in the mitochondria of other tissues (Schneider<sup>9</sup>) but not yet so thoroughly investigated.

It is clear from this study that it would be a difficult matter to specify at present the optimal conditions for the muscle ATPase activity. Further it has to be remembered that there is considerable evidence (to be discussed later) for localization of materials in the muscle fibre. This applies to the adenylic compounds and to inorganic salts, so that we cannot assume that the ionic concentrations where the enzyme is acting *in vivo* are the same as the overall ionic concentrations. Nor have we data from which to gauge the extent of  $p_H$  variation within the fibre.

### THE TIMING OF ATPASE ACTIVITY in vivo AND THE EFFECT OF ATP ON MYOSIN

We come now to the timing of the ATPase activity: does it occur simultaneously with contraction or with relaxation? With this is bound up the whole question of the details of interaction between myosin and ATP. Does ATP enter into combination with myosin as a result of the stimulus or is it always in some kind of combination with some part of the myosin chain? Does the ATP in combining with the myosin act as a trigger to set off the energy liberation and the shortening of the myosin? Do tension development and work performance depend on simultaneous ATP breakdown? Or does the energy liberated in contraction come in the first place from energy stored in the myosin chains, the energy from ATP dephosphorylation being used during relaxation to reconstitute the chains in their initial state?

None of these questions can be answered with assurance. We shall consider briefly the results obtained from experiments *in vitro* on the effect of ATP on myosin and actomyosin since it is from further pursuit of such analytical procedures that we can best hope to get a clue to the intimate mechanism of contraction. But at the present time perhaps the best indication of an answer to any of these questions comes, not from any results *in vitro* but from the fact that, in the living muscle, relaxation gives the impression

of being an active process. For example, during onset of fatigue, it is the relaxation phase which becomes slowed rather than the contraction phase. This would suggest an answer in the affirmative to the last question.

### THE EFFECT OF ATP ON MYOSIN SOLS

The experiments, during 1941 and 1942 of J. Needham and his collaborators in Cambridge<sup>10</sup> and of Szent-Györgyi and his collaborators in Szeged<sup>11</sup> showed the highly specific reversible effect of ATP in diminishing the double refraction of flow and the viscosity of solutions of myosin (prepared in the classical way) in 0.5 M KCl. The decrease in the length to breadth ratio of the micelles thus indicated was traced by the Szent-Györgyi school to the splitting of actomyosin; and the isolation of the new muscle protein, actin, by Straubl<sup>12</sup> followed.

In a recent publication, JORDAN AND OSTER<sup>13</sup> have described experiments on the light-scattering properties of solutions of classical myosin in 0.5 M KCl before and after addition of ATP; they interpret their data on the change in ratio of forward to backward scattering as showing an increase in coiling of the protein particles, these being present, before ATP addition, in the form of slightly coiled rods.

The validity of this interpretation depends upon the presence of the actomyosin in the solution in the form of discrete rods and not in the form of the branching network to be seen in electron micrographs. It is very possible that the dilute solution used did contain rod-shaped particles especially as it had been subjected to ultra centrifugation, which might be expected to carry down the network.

An increased coiling of such actomyosin particles (or of myosin particles formed from them) under the influence of ATP would obviously be of importance in considerations of muscle contraction and further work along these lines, including observations on pure myosin (myosin A), will be of much interest.

It is a matter too, for future experiment to decide whether evidence for the increased coiling of the rods (after they are set free from the network) can be obtained from electron micrographs. So far attention has been concentrated on the behaviour of the network with ATP and the appearance of the resulting débris has not been closely studied.

Another step forward in our knowledge of the interaction of myosin, actin and ATP was gained by the observations of Bailey and Perry<sup>14</sup> on the effect of -SH reagents. They showed a close correlation between the effect of reagents which oxidize or combine with -SH groups in inhibiting ATPase activity of myosin on the one hand and its power to combine with actin on the other. Thus certain -SH groups of myosin are necessary for its combination with ATP (and this is in line with much other information about enzymes concerned with ATP). These same -SH groups are necessary for combination of myosin with actin, and if ATP is added to actomyosin it displaces actin from these groups and itself combines. These results are important, not only in throwing light on the mechanism of the dissociation effect. The earlier experiments of the Needham and Szent-Györgyi groups had indicated an interaction between ATP and the protein responsible for the double refraction of flow and the high viscosity; that is to say, they made it unlikely that ATPase activity of the myosin preparations was to be put down to presence of small amounts of some other protein. This line of argument is strongly re-inforced by the work of Bailey and Perry which forms the best evidence so far

for the ATPase activity of myosin itself. The knowledge gained from the study of interaction between ATP and myosin sols must clearly play a useful part in our progress towards understanding of muscle contraction. But it does not seem that any deductions having a direct bearing on the question we have raised can be drawn from it at the moment. Certainly a deduction recently made from the results of Dainty *et al.*<sup>15</sup> by Morales<sup>16</sup> that "the catalytic activity of ATPase, that is of acto-myosin, rises exponentially with disorientation of the protein" is not justified.

#### THE EFFECT OF ATP ON MYOSIN THREADS

The similarity in rod and intrinsic birefringence and in the X-ray diagram between artifical myosin threads and muscle fibres led to hope that important progress might be made by study of the effect of ATP upon such threads; especially since it was found that they still retain ATPase activity and could withstand a certain amount of tension (Engelhardt<sup>17</sup>) without breaking.

ENGELHARDT used threads made from classical myosin and containing about 2% protein. Subjected to loads of about 200 mg such threads show a reversible extension. If the threads are tested, immersed not in KCl solution but in 0.005 M ATP, this extensibility is increased by 50–100%.

This effect of rise in extensibility with loaded threads is in contrast to the striking shortening effect obtained by Szent-Györgyi<sup>11</sup> with unloaded actomyosin threads (myosin B), suspended in dilute (0.05 M) KCl. Addition of ATP (0.002 M) led to isodimensional contraction, with shortening up to 66%. This shrinkage of the actomyosin thread is accompanied by great loss of water, the percentage falling from about 97 to 50.

The observations of BUCHTHAL et al. 18 form a link between these two sets of observations. Using actomyosin threads (which had been dried to a N content of 16.15% and then allowed to imbibe water for 30 minutes from 0.9% NaCl solution) they found that addition of 0.002 M ATP caused a 30% shortening of the unloaded thread; while with a load of 110 mg there was an increase in length of 30%. Even so small a load as 5 mg caused a slight lengthening.

PERRY et al.<sup>19</sup> have contributed an instructive electron microscope and X-ray study of the synaeretic effect of ATP on actomyosin gel in 0.05% KCl. The photographs of the control gel show a dense tangled network. After ATP addition, the network has opened out; it would appear that small linear fibres are first formed (as might be expected on a splitting to actin and myosin) and that these aggregate side by side to form denser fibres. The X-ray diagrams from the same material show no fundamental difference between actomyosin before and after synaeresis. These observations were taken to indicate that there is no increased intramolecular folding with intramolecular synaeresis, but rather that the water loss is intermolecular accompanied by lateral aggregation.

When all these facts are considered together it seems that the discrepancy originally felt between the results of Engelhardt and of Szent-Györgyi disappears. The effect of the ATP in both cases is to cause breakdown of the actomyosin network followed by aggregation of the particles and squeezing out of water. When the thread is loaded, the fall in elasticity consequent upon the disappearance of the network is the obvious aspect; when the thread is unloaded, this aspect is not noticeable but the shortening due to synaeresis can manifest itself.

BUCHTHAL et al. 18 have reported that treatment of fresh actomyosin threads (3%

protein) with the sulphydryl reagents iodoacetate and porphyrexid causes decreased shortening when ATP is subsequently added. The interpretation of these results is not immediately obvious for, when sulphydryl reagents are added to actomyosin sols in 0.5 M KCl, there is a decrease in viscosity as would be expected if the reagent, like ATP, broke the link between the actin and the myosin. The effect is slower than with ATP itself, probably because the reagents react also with other –SH groups (for example, in the actin) while the ATP reacts specificially with the connecting groups. In the case of the threads, there seems no reason why –SH reagents should inhibit the splitting of the myosin from the actin; possibly the attachment of ATP to the myosin, as well as the presence of free –SH groups, is involved in the further changes in state of aggregation and it is these which become impossible.

It seems doubtful whether these phenomena of synaeresis are connected with the mechanism of contraction. If the removal of water is intermolecular, this would lead in vivo to a narrowing rather than a shortening of the fibres, since both myosin and actin are known to be arranged with their long axes parallel to the fibre axis. Perry et al. 19 have remarked on this and also pointed out that though the loss of water associated with volume contraction is very rapid, the reverse process (which might be analogous to relaxation) is slow and there is little information as to its degree.

#### THE LOCALIZATION OF SUBSTANCES IN THE STRIATED FIBRE

The anisotropic (A) band seems to have had, from the early days of work on muscle fibres, a particular interest for observers. In spite of the many variations and discrepancies of description of the histological appearance of contracted muscle (depending on the different sources of the muscle; differences in preparation, whether fresh or fixed and stained; differences in optical set-up; and differences in degree of contraction) there has been a widespread if by no means unan mous opinion that it is the A-band which becomes shorter in appearance on contraction of the muscle while the I-band may show little change or even become longer.

This conclusion was probably based partly on the formation of contraction bands (see below) in strong contraction; a condition where the position of the staining material has actually become reversed with respect to the A- and I-bands; but studies like those of Buchthal et al.<sup>20</sup> on single living fibres do show a decrease in the A/I ratio in early contraction. The work of Buchthal et al. was quantitative and showed, in short isometric tetani, a decrease in length of the A-band of 18%, an increase in the I-band of 28%.

The visible changes in length of the A- and I-bands have often been taken as indicating that the actual contractile process was limited to the A-bands; the I-bands, though not necessarily considered as passive, being the seat of less important changes. The conception of more recent years took the form that in the A-bands the protein micelles undergo folding while this process is much less or gives place to unfolding in the I-bands.

The idea that the protein of the A-band actually differed in kind from that of the I-band was given up as more accurate estimates of the myosin content of the muscle became available. For several years the view was then prevalent that the fibril consists of collections of myosin chains, arranged in the anisotropic bands with their long axes parallel to the fibril axis, but in the isotropic bands having much less orderly arrange-

ment. However the recent production of electron micrographs of muscle fibres (Hall, Jakus, and Schmitt<sup>21</sup>), showing continuous micelles passing straight through A- and I-bands, brought the realization that the lack of orderly arrangement in the I-bands (if it exists) must be at a level of dimension below the resolving power of the electron microscope. The work of Dempsey et al,<sup>22</sup> about the same time, demonstrated the presence in I-bands of lipoids with negative double refraction and the possibility of converting striated fibrils, by thorough extraction with fat solvent, into fibrils of uniform positive double refraction. Matoltsy and Gerendas<sup>23</sup> also report experiments indicating the presence of a substance of negative double refraction in the I-band.

The present-day conception is therefore rather that the fibrils consist uniformly

The present-day conception is therefore rather that the fibrils consist uniformly through their length of bundles of myosin (or actomyosin) chains pursuing an apparently straight course, and as far as our knowledge of these chains goes, there is no obvious reason for a localization of the contraction process in the A-bands.

We have some further knowledge indicating a high degree of localization of other substances within the fibre, and also (a matter of particular interest) in some cases suggesting a change in location during contraction. Since the possibility arises that changes in position of non-myosin material may affect the visible length of the A- and I-bands, this subject may be pursued a little further. Thus there is good evidence (Caspersson and Thorell<sup>24</sup>) that material with selective absorption at a wave-length of 265 mu is concentrated in the I-bands in resting muscle; in muscle after vigorous contraction there is spread of the material into the A-bands. The adenylic compounds are the most likely to be responsible for this effect; it has also been suggested that they may contribute to the negative double refraction of the I-band (Barer<sup>25</sup>).

Then we have the more recent work of Scott and Packer²6 (using a rapid freezedrying method and careful avoidance of water to prevent movement of soluble salts) confirming a good deal of earlier work in finding the greater part of the ash in the Aband. There were indications that this localization applied to calcium and magnesium. Finally it has long been known that the A-bands contain material which stains deeply with basophilic dyes. This material seems to contribute to the dark colour of the A-band in fresh fibres in ordinary light, but does not seem to be concerned with the double refraction of the A-band. Histological literature abounds with detailed descriptions of the movement of this material (the A substance) during contraction. Such descriptions are usually concerned with fixed and stained material but as of more interest we may take the example of the more recent work of Speidel²7 on living muscle of vertebrates and invertebrates. He describes, as the fibre shortens, first a shortening of both A and I; secondly a blurring of cross striae when the sarcomeres have shortened by about one third, as if the dark refracting material were undergoing profound redistribution or chemical change; thirdly, concentration of the dark refractive material (the contraction band) about each Z disc (crossing the centre of what was, during rest, the I-band).

It is interesting that the electron microg aphs of Hall, Jakus, and Schmitt<sup>21</sup> show material (of which we know only that it has high electron-scattering power and high affinity for phosphotungstic acid) concentrated in the resting fibril in the A-band. When fibres are stained with phosphotungstic acid and fixed in different stages of contraction, stages can be made out in the electron micrographs indicating the spreading of stainable material from the A-bands, until at about 40% shortening a state is reached with a narrow dense band in the position of the Z-membrane, the rest of the fibre, including the A-band, being uniform with comparatively faint staining. The close

correspondence with the behaviour of the "A" substance described above is striking. These observations may be summarized as follows:

TABLE I

| I-Band                        | Movement during contraction | A-Band                                                                                          |
|-------------------------------|-----------------------------|-------------------------------------------------------------------------------------------------|
| Lipoids<br>Adenylic compounds |                             | Salts, perhaps especially Ca and Mg<br>Basophilic A substance<br>Electron scattering substances |

It seems that there must be some intimate connection between the three classes of substance mentioned in the 3rd. column; whether the same substances are actually responsible for the staining and the electron-scattering phenomena we do not know.

Besides these localizations which have been recognized, and which must have significance for contraction it seems likely that there may be much localization still unknown. In particular it is to be expected that the soluble protein fractions, myogen and globulin X, including most of the enzyme equipment of the muscle, instead of being merely dissolved in the sarcoplasm, will show pattern.

#### CONCLUSION

If one is to make any sort of tentative picture of the mechanism of contraction, one must, under present conditions, be allowed a bias towards one side or the other in answering the question "Is relaxation of the fibril an active process, requiring provision of free energy?" The writer would like to take the standpoint that an affirmative answer best fits the observed physiological behaviour during relaxation and that observations on the relations of heat production and on the effect of work on heat production are not at variance with this view.

One can make the basic assumptions that, in the stimulated muscle, chemical reaction becomes possible between groups situated along the protein chain; that this reaction goes on with production of free energy and that in the resting muscle there is some configurational hindrance to its taking place. Further, one can assume that the number of these groups which can react together will depend upon the length which the muscle is made to assume, being fewer at greater lengths and increasing in number as the muscle shortens. It is known that during a twitch the amount of "shortening heat" production is proportional to the degree of shortening of the muscle, while the rate of "shortening heat" production is dependent on the speed with which the muscle shortens, (A. V. Hill<sup>28</sup>). Thus for shortening a given distance, the "shortening heat" production is the same, whether the shortening is slow or fast. But the rate of shortening depends on the load, being slower the greater the load; thus at slower rates of shortening between two given lengths, more work is done and more energy must be produced, since the heat remains the same. If this energy production is the result of the interaction of the same groups at different rates of shortening, we must suppose that, at the slower rates, repeated interaction takes place. When speculations are made as to the timing of ATP breakdown, it is usually supposed that this is confined either to the contraction phase or to the relaxation phase (in the latter case its energy being used to restore energy-rich protein linkages). If we suppose that, when work is done, before a pair

of groups can react together a second time, they must have been put back into their original state by means of free energy provided by reaction with ATP, we see that ATP breakdown could begin within the contraction phase, even though it were associated with restoration of the chains.

A. V. HILL<sup>29</sup> has shown that the relaxation phase of a twitch is free from heat, if the work done is not allowed to degenerate into heat. During this period, on the view under discussion, ATP breakdown would be continuing the process of separating the reactive groups, a process leading now to the lengthening of the fibril. Since no heat is associated with the relaxation phase, this process would seem to be 100% efficient, and the waste heat associated with the contraction phase would appear to be due to the primary reaction along the protein chains. As we have seen, the anaerobic recovery process (immediately following an anaerobic contraction) is known to go on with very little heat wastage; it is not unlikely that there is a similar efficiency in the relaxation process. A mechanism suggested for the transfer of energy (Kalckar<sup>30</sup>; Dainty et al.) is the transfer of phosphate from ATP to the protein chains; this still remains a possibility, (see F. Buchthal et al.30).

The Verkürzungsort still retains its mystery but we begin perhaps to see in what direction solution lies.

I am indebted to Dr K. Bailey and Professor W. T. Astbury for the benefit of discussion with them.

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# A CONSIDERATION OF EXPERIMENTAL FACTS PERTAINING TO THE PRIMARY REACTION IN MUSCULAR ACTIVITY

by

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One of the most significant results of the investigations of Meyerhof and his associates was the demonstration that, of all known metabolic processes the splitting of adenosine triphosphate (ATP) is most directly connected with the fundamental mechanical event in contracting muscle (Lohmann<sup>17</sup>; Meyerhof<sup>20</sup>; see<sup>27</sup>, Chapter II). Notwithstanding its importance this result is subject to two limitations. For one thing, the nature of the breakdown of ATP is still not yet satisfactorily settled; the assumption now popular that it is due to a straightforward hydrolysis by the enzyme myosin-ATPase leads, at the present state of knowledge, to difficulties. On the other hand, the introductory statement as well as Lohmann's original conclusion contained the restriction "of all known metabolic processes". It is possible that ATP, before becoming decomposed, engages in other more intimate reactions with the contractile structure, as will be emphasized in this paper. These restrictions do not diminish, they rather enhance the emphasis on ATP, and it is exactly here that the most direct link between the study of muscular metabolism and the modern analysis of its function exists.

An essential contribution to this latter category has been made by SZENT-GYÖRGYI<sup>35</sup> by his discovery of the contractility of actomyosin, his biochemical analysis of the components of this complex substance, and by the study of various aspects of its behaviour. This work has repeatedly been summarized in greater or lesser detail (*l.c.*<sup>36</sup>, <sup>37</sup>, <sup>38</sup>; <sup>27</sup>). There are,however, a few points which may be discussed as a suitable introduction to the problem of this essay.

If ATP is indeed the ultimate action substance of muscle, as SZENT-GYÖRGYI in logical continuation of MEYERHOF's work assumes, it is to be expected that addition of this compound to a muscle will evoke contractions. This has been achieved. Contractions were obtained by BUCHTHAL et al.<sup>2, 5, 6</sup> by close arterial injection of ATP, and by its application to isolated muscle fibers. The latter effect was also studied in a quantitative manner by ROZSA<sup>32</sup>, using a different method. Since BUCHTHAL finds the effect to persist after curarization, it may appear difficult to assume an indirect stimulation. Nevertheless, the possibility that ATP in such experiments activates the excitatory process of the muscle, rather than the contractile structure directly, has to be kept in mind. Rozsa's results indeed suggest this to be the case. Since the excitatory process in its turn activates or liberates the ATP present, this BUCHTHAL-Rozsa effect may play an essential rôle in the conduction of the contraction wave.

A simpler and more convincing system is what the writer proposes to call the fibril preparation, which has been introduced by SZENT-GYÖRGYI<sup>35</sup>, I, page 24. Its great

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significance has been underlined by MEYERHOF<sup>21</sup>. If a muscle with parallel fibre arrangement is kept in distilled water for a prolonged time, and is frozen and thawed, one obtains a preparation which consists essentially of the original undisturbed fibrils, and from which the soluble constituents of the sarcoplasma, including all factors which have to do with irritability, have been removed. No stimulation will cause contraction of these fibrils. They shorten, however, promptly if ATP in a proper electrolytic medium is added. In this case there appears to be little doubt that ATP has directly acted upon the contractile structure itself.

The analysis has gone further. One can extract and fractionate the muscle, and obtain a crystalline protein, myosin (SZENT-GYÖRGYI, l.c.) and supposedly pure actin (Straub<sup>33, 34</sup>). Combined with each other, they form the complex actomyosin which can also be extracted directly (SZENT-GYÖRGYI, l.c.) and from which threads may be spun. These threads, suspended in the same solution of KCl and MgCl<sub>2</sub> as is used with the fibril preparation, will contract in response to the addition of ATP (SZENT-GYÖRGYI l.c.). It is true that these threads, unlike fibrils, become shorter and thinner instead of thicker. This is however merely a consequence of the fact that the actomyosin particles in such a thread are very imperfectly orientated. After initial difficulties (Gerendas 13), Buchthal et al.3 have succeeded in preparing well orientated threads, and these behave in accordance with the rule by becoming thicker during contraction. Two objections have been made. Buchthal et al., at the International Congress of Physiology in Oxford (1947)3 (repeated by Perry et al.29) raised the difficulty that such threads, when loaded, do not contract but become stretched upon addition of ATP. This may be due to the circumstance that in the formation of the threads very few and weak points of intermicellar attachment are formed, which are not able to carry any strain. Since the action of ATP upon actomyosin includes a disaggregative effect as well, the plasticity of the threads is actually increased by ATP. In the fibrils on the other hand, very strong intermicellar bonds exist in the densely packed system. The second objection, made by ASTBURY at the Experimental Cytology Congress in Stockholm (1947) (PERRY et al. 29), was that upon electron-microscopical investigation actomyosin, after treatment with ATP at 0.05 M KCl, 0.005 M MgCl<sub>2</sub>, showed a dispersion of the original aggregates, with no indication of a true contraction. Since however after the addition of ATP, during the drying of the preparation, the salt concentration had to increase and pass the limit above which the actomyosin dissolves and disaggregates, this experiment has no bearing upon the mechanism of contraction. Finally, the same authors<sup>29</sup>, (p. 677) object that, even if the shortening of actomyosin threads may imitate the contraction of muscle, these threads show no relaxation. According to all we know about muscle, however, relaxation would seem to be the more complicated phenomenon. That this has not yet been reproduced in vitro is no objection against a contribution relevant to contraction. The objection is invalid the more so, since the contraction process in threads takes place to an extreme extent. Such extreme shortenings are irreversible even in vivo (RAMSEY's deltastate<sup>31</sup>). It seems thus that Szent-Györgyi's observations on the effect of ATP upon actomyosin are not subject to any serious inconsistency at this moment.

A further simplification may be achieved by working not with carefully prepared actomyosin threads, but with a suspension of finely precipitated actomyosin flocks. Addition of ATP will cause their contraction as well. Since they are perfectly disoriented, their contraction will take place in all dimensions equally. It is manifested by an increased tendency of the flocks to settle (SZENT-GYÖRGYI'S "superprecipitation"), and

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its extent can be quantitatively established by determining the volume of the gel pellet after centrifugation (Mommaerts<sup>22</sup>). One can thus study contraction at various levels of subcellular and supermolecular organization.

A still simpler system is a solution of actomyosin in 0.5 M KCl. As Szent-Györgyi has discribed<sup>35, 37</sup>, the high viscosity of such a solution is greatly decreased by ATP. The analysis of this effect has shown that it is not due to a contraction of dissolved actomyosin micells<sup>22, 23, 24, 25</sup>. The true reason, as is well established now, is a disaggregation of the actomyosin into its components, myosin and actin. Although the immediate connection between this disaggregation and the contraction at lower ionic strengths is not clear, it may be presumed that the first effect of ATP is identical in both cases. One of the aspects of this first effect apparently is an elimination of certain intermolecular bonds. In the case of dissolved actomyosin, which is on the verge of disaggregation, the complex falls apart. At low salt concentration, where more or other bonds may exist, this dissociation cannot reveal itself, but the contraction can. It appears unlikely that in solutions of actomyosin contraction takes place side by side with the disaggregation. For it is an empirical fact (Szent-Györgyi, l.c.; Erdös<sup>11</sup>) that without actin myosin cannot contract; in 0.5 M KCl solution, ATP separates the actin and myosin so that no contractile complex then exists. Although the relation between the two effects is not understood, the study of the disaggregation in solution is highly useful, for it enables a great variety of experiments to be performed which would not be possible in strongly heterogeneous systems. As a result of the study of this viscosity effect, mainly three

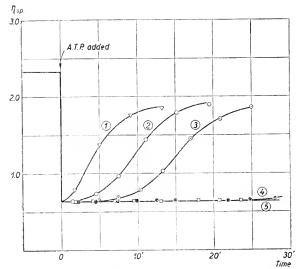


Fig. 1. Effect of ATP upon the viscosity of an actomyosin solution. At zero time, ATP is added. In all experiments, 2.5 mg actomyosin were present per ml, dissolved in 0.5 molar KCl at neutral reaction. Curve 1 ( $\triangle$ ) refers to an experiment in the presence of 0.001 molar CaCl<sub>2</sub>, curve 5 ( $\square$ ) to an experiment with 0.001 molar MgCl<sub>2</sub>. The amount of ATP added was 25·10<sup>-8</sup> moles in the experiments 1, 2 and 5; 50·10<sup>-8</sup> in 3; 200·10<sup>-8</sup> in 4 (see text).

conclusions seem possible:

First, the effect is fast. It apparently takes a fraction of a second to reach completion. Methods for the exact study of its time course have not yet been available.

The second conclusion needs more elaborate explanation<sup>25</sup>. Fig. 1 shows a few examples of the viscosimetric measurement of the effect of ATP upon an actomyosin solution. It will be seen that, after the initial viscosity response a recovery effect sets in, which takes more time the more ATP had been added. It is inhibited by Mg+ and activated by Ca+-ions, and is to be identified with the removal of the ATP by the ATPase associated (Polis and MEYERHOF<sup>30</sup>) with the myosin. The viscosity response itself is not inhibited by Mg+ and activated by Ca+ (rather the opposite) and can also take place if no hydrolysis occurs. Hence the second conclusion: the

effect of ATP upon the aggregation of actomyosin is not caused by any known breakdown

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of the ATP. In an attempt to specify the nature of this primary reaction between ATP and actomyosin, the quantitative relation between the amount of ATP added and the

magnitude of the physical effect has been studied22, 25. Because of § particular experimental difficul-  $\frac{1}{8}$  109 ties, the results have not yet been satisfactory, but an example as that of Fig. 2 shows that one has to assume the formation of a sparingly dissociated compound between ATP and (acto)-myosin. Further quantitative researches are in progress. The relationships depend on whether Mg+ or Ca+ are present and the best result in the presence of the promoting Mg+ showed that I mole of ATP causes the maximal change in as much as 300000 grams of myosin. The dotted line (Fig. 2) represents what would be expected if the ATPactomyosin complex would be completely undissociated; the deviation of this from the actual curve is possibly still less than is indicated by the results, which are obtained by difficult measurements in a rapidly changing system. The third conclusion reads therefore: the effect of ATP upon a measured

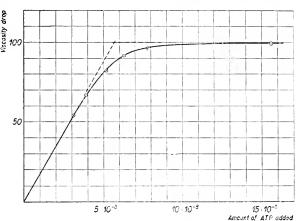


Fig. 2. Dissociation curve of the ATP-myosin complex. The effect of varying quantities of ATP upon the magnitude of the viscosity drop (at o°; extrapolated to zerotime) of actomyosin was studied. System: 20 mg actomyosin in 10 ml 0.5 molar KCl, 0.02 molar MgCl<sub>2</sub>. Abscissa: amounts of ATP added to this system. Ordinate: viscosity drop, expressed as percentage of the effect obtained with a large excess of ATP. The dotted line, tentatively drawn as representing complete absence of dissociation, indicates that the maximal effect is reached when 5.7·10-8 moles ATP combine with 20 mg actomyosin, corresponding to 1 mol ATP per 300000 g myosin. The difference between the dotted and the experimental line indicates that at half-equilibrium the concentration of free ATP is much less than the total ATP concentration of 3·10<sup>-6</sup> moles per liter (see text)

physical property of actomyosin is due to the formation of a sparingly dissociated ATP-(acto-) myosin complex. One is led to a similar conclusion by studies of the same combination in heterogeneous, contractile actomyosinsystems, but I had insufficient opportunity to study this in full detail. In solution, the measured effect was maximal when I mole of ATP was present for 300000 gram myosin. It is possible that upon addition of more ATP, more is bound and stronger physical changes are induced. This cannot be measured in solution, but may possibly be found in further studies with different methods. Not more can thus be stated than that 300000 gram myosin combine with at least I mole of ATP, or roughly that 100 mg myosin, present in one gram of muscle, combine with  $3 \cdot 10^{-7}$  mole ATP or more.

Naturally, the mere demonstration that ATP, when interacting with actomyosin, actually combines with it (most probably with the myosin component only), is yet no explanation of the mechanism of its action. In this connection, the question arises whether ATP is only bound to myosin, or whether any further reaction takes place between them. More precisely the question may be asked whether myosin is phosphorylated by ATP. The author spent a summer trying to demonstrate such a phosphorylation. Actomyosin and ATP were allowed to react in a proper medium, and were

then separated by centrifugation. Considerable quantities of P were found in the precipitate. Eventually it was found out, however, that the apparent phosphorylation was proportional to the amount of calcium in the system, and what appeared to be a phosphorylation turned out to be nothing else than a coprecipitation of actomyosin, Ca<sup>+</sup> and inorganic phosphate, the latter being formed by enzymatic splitting of the ATP\*. It is true that without Ca<sup>+</sup> very small amounts of P were found in the sediment, but those were neglected at that time.

Meanwhile, however, Buchthal, Deutsch et al.<sup>4</sup> conducted their study of just this small effect. They find amounts of about or above 15  $\mu$  gram P per 100 mg myosin (Professor Buchthal kindly provided me with additional data not given in the preliminary paper), which would correspond to  $5\cdot 10^{-7}$  mole or more of P transferred to 100 mg myosin (1 gram muscle). This is the same order of magnitude as that of the combination between ATP and myosin. Indeed, Buchthal, Deutsch et al. also measured an uptake of nucleotide. It thus seems likely that the primary reaction between ATP and myosin does not remain restricted to a mere combination, but is followed by more intricate interactions as well.

In spite of the insufficient information available, some further quantitative aspects of the (acto-)myosin-ATP dissociation curve just referred to may be discussed. We indicate the molar concentrations of the myosin (taking the relative weight of the unit combining with one ATP), the complex, and the ATP with  $c_M$ ,  $c_{MA}$  and  $c_A$ . From viscosity measurements, as described above, it would be possible to determine the value of K, most easily by measuring the  $c_A$  at which half the maximal viscosity response is obtained (for  $c_{MA} = c_M$ ,  $K = c_A^{-1}$ ). This problem is now under investigation, but previously no values for  $c_A$  have been obtained due to experimental difficulties. Naturally, only the concentration of *free* ATP is relevant here; Engelhardto (page 189), who attempted to calculate an equilibrium constant from my earlier measurements<sup>22</sup> erroneously took the total ATP amount present in the system. If the total ATP concentration is below  $10^{-5}$ , (see Fig. 2)  $c_A$  is very much smaller, perhaps around  $10^{-8}$ . Thus, K will be of the order of  $10^7$  or more, and the value of RT ln K will be in the range of 10000 calories, a very considerable free energy effect.

There is an independent way of estimating the quantitative relationships between ATP and myosin in a single elementary contractile event. As is well known (comp. Lundsgaard, l.c.), in iodoacetate poisoning, where the muscle uses up its stores of ~ P, some seventy contractions are possible. Such a muscle, before beginning its activity, contains some  $2.5 \cdot 10^{-5}$  moles of  $\sim$  P per gram, counting only the terminal P of the ATP. One can look upon every twitch as one elementary event involving a fraction of this ~ P in the form of ATP, which first combines with myosin, and is thereupon decomposed. For simplicity of argument, it will be assumed that the poisoned muscle performs some 50 full, rather than 70 decreasing twitches. Since  $2.5 \cdot 10^{-5}$  moles  $\sim$  P enable to 50 full twitches, one elementary event involves the reaction of  $5 \cdot 10^{-7}$  moles of  $\sim P$ with the contractile structure, followed by direct or indirect degradation into inorganic phosphate. Since this same amount of muscle contains nearly 100 mg myosin, it is found that in every complete elementary process 1 mole of  $\sim$  P reacts with 200000 gram myosin. This value is so close to the proportion of I ATP to I-3 hundred thousands myosin which I regularly found in vitro that it would be hard to consider it as a mere coincidence.

<sup>\*</sup> The critical attitude of Dr. Gerhard Schmidt is gratefully acknowledged.

It is still difficult to judge the exact physiological meaning of the described reaction, but it is of obvious interest to see whether a theory ascribing to it the significance of the primary event in muscular activity would meet the standards set by Hill's thermal measurements. As is well known, a single anaerobic twitch, in which the primary event would take place only once, is accompanied by an appearance of about  $3 \cdot 10^{-3}$  calories per gram muscle<sup>14</sup>. In the given picture, this primary event would involve the combination of  $3 \cdot 10^{-7}$  or more moles of ATP with the structure protein. Thus the heat effect of this combination per mole ATP would have to be 10000 calories or less. This has not yet been measured, but the requirement seems to be quite in line with what could be expected. It seems a permissible hypothesis therefore to identify the primary event of contraction with a combination and further reaction between ATP and (acto-) myosin.

We shall now turn to a discussion of the chemical basis of relaxation, and will have to correlate this event, by exclusion, with the enzymatic breakdown of ATP or its myosincomplex. In connection with the close association between ATPase and myosin, the current assumption is that it is the myosin-ATPase itself which hydrolyses the ATP, and thus makes the energy of this reaction available to the contractile structure. After an extensive study of the activity of myosin-ATPase it has been estimated26 that in muscle the overall speed of hydrolysis by this enzyme can amount to only about 3.10-3 mg P per mg myosin per minute. The actual speed of ATP breakdown in active mammalian muscle is much higher. From Lundsgaard's results with frog muscles the writer estimated the speed of this process to be around 2·10<sup>-1</sup> mg P per mg myosin per minute, and a reinvestigation of all relevant data (27Chapter III) gave rise to the same or even higher values. Likewise, Braverman and Morgulis<sup>1</sup> essentially confirmed these results and reported the same disproportion. To reformulate the difficulty: the actual speed of breakdown of ATP in active muscle proceeds a hundred times faster than the myosin-ATPase under the given circumstances can account for. Several explanations of this discrepancy seem possible. Either, intact muscle contains unknown potent activators of the myosin-ATPase. Or, the true reaction is not at all a hydrolysis of ATP, but a phosphorus transfer to some acceptor; in fact there are indications (Lundsgaard<sup>18</sup>; CORI AND CORI7) that a P-transfer of ATP to fructose-6-phosphate under formation of hexose-diphosphate is a significant reaction. Further, it is not yet possible to judge which rôle the new ATPase described by Kielley and Meyerhof<sup>16</sup> has. Several possibilities for a solution of the dilemma thus seem to exist, and the identification of the exact course of ATP breakdown may throw a significant light upon the question of relaxation. At this moment however, no suggestions seem to be indicated.

The above considerations have been developed on the basis of *in vitro* experiments only, and the task remains of identifying the sequence of events in the contraction cycle of a living muscle. In this field, we owe most direct and illuminating experiments to Dubuisson<sup>8,9</sup>, who studied the rapid p<sub>H</sub> changes which accompany a contraction. It was found that first an acidification occurs which in favourable specimens was preceded by a small reaction change in the opposite direction. Then there is an alkalinization, followed in turn again by an acidification. The last two changes could be identified convincingly: they are due to the dephosphorylation of phosphocreatine, and to the formation of lactic acid. The latter process takes place only after the mechanical events, the former is coincident with the relaxation. The initial acidification is correlated with the initiation of the contraction process, and is therefore of great interest. Dubuisson assumes it to be due to hydrolysis of ATP, but this conclusion is tentative; acidification

might likewise be caused by the binding of ATP by myosin followed by phosphorylation of the latter. On this point therefore, no decision seems possible as yet.

With respect to the moment at which the energy of metabolism is made available to the contractile apparatus, it is now customary (see<sup>27</sup>) to distinguish two possible mechanisms. In the first of these, chemical energy may be transferred at the very moment of contraction, when it is necessary. The alternative possibility is that the primary event merely releases, by a trigger action, a spontaneous contractile process (often paralleled with the shortening of stretched rubber), and that it is the event of relaxation which is linked with exergonic metabolic reactions in order to restore the active state. The latter category, the so called postenergization mechanisms, seems difficult to reconcile with the results of Fenn and Hill<sup>12, 15</sup> indicating rather the existence of contraction-coupling. Nevertheless, postenergization hypotheses are rather in demand at present, and the opinion seems to prevail that Szent-Györgyi's work may lead to this type of inter probation, a viewpoint taken, e.g., in the speculations of Morales<sup>28</sup>. As the present communication shows, the analysis of the effects discovered by Szent-Györgyi gives, on the contrary, rise to a preenergizationtheory.

It has been the purpose of this discussion to show where the actual experimental analysis of the contractile event, in terms of ATP-actomyosin interaction, at present stands. No detailed theory seems warranted, or, as Meyerhof said in 1930<sup>19</sup> (p. 280): "Es soll daher hier weniger eine bestimmte Theorie ausgearbeitet werden als die festgestellten Tatsachen und die sich daraus ergebenden mehr oder weniger wahrscheinlichen Folgerungen zusammengefasst sowie Missverständnisse gegenüber der Auslegung dieses Tatbestandes beseitigt werden". But neither should the impression prevail that "... (man) auch heute eigentlich noch gar nichts weiss".

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# SOME FACTORS INFLUENCING THE CONTRACTILITY OF A NON-CONDUCTING FIBER PREPARATION\*

by

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#### INTRODUCTION

One of the most important contributions of Otto Meyerhof was the discovery of the high energy which may be contained in phosphorylated compounds. Following the description of phosphocreatine (phosphagen) by FISKE AND SUBBAROW<sup>1</sup> and EGGLE-TON AND EGGLETON<sup>2</sup>, MEYERHOF found, in 1927, that the enzymatic decomposition of this compound is connected with the liberation of a large amount of heat<sup>3</sup>. The energy released is about 10000 to 12000 g calories as compared with 2000 to 3000 of other phosphorylated compounds, e.g., hexose mono- and diphosphate, pentose and triose phosphate and other esters, i.e., all those compounds where the phosphate is linked to an alcoholic hydroxyl. Meyerhof found a similar high energy in argininephosphate which in many invertebrates takes the place of creatinephosphate<sup>4</sup>. A few years later, when in his laboratory, LOHMANN had isolated adenosinetriphosphate (ATP) from muscle, Meyerhof<sup>5</sup> showed that about 24000 g calories are released by the breakdown of ATP to adenosine monophosphate (AMP). This is about the same amount of energy for each of the two P as that derived from the P of phosphocreatine. Soon afterwards, two more phosphorylated compounds, intermediates in glycolysis, were found to be rich in energy: phosphoenol pyruvic acid<sup>6</sup> and 1.3-diphosphoglyceric acid, isolated by Nege-LEIN AND BROMEL in WARBURG'S laboratory. The great significance of MEYERHOF'S discoveries of energy-rich phosphates for the understanding of intermediate metabolism and the far reaching implications have been reviewed in this country by LIPMANN<sup>8</sup> and Kalckar9.

Among all the energy rich phosphorylated compounds, ATP plays a special rôle. Originally the study of this compound was limited to the glycolytic cycle. More recent studies, however, have shown that ATP has a more general importance, as the source of energy in intermediate cellular reactions, as e.g., acetylation (Nachmansohn¹o), urea formation (Ratner¹¹) and many others. Although the essential rôle of ATP in intermediate metabolism becomes continuously more evident, its function in the muscle cell in which it was first discovered and studied is still one of the most challenging problems to biologists. From the work of Meyerhof and his associates, it appeared likely that ATP was involved in the primary changes of the protein during muscular contraction. No other

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chemical reaction is known to be more closely associated with the contractile mechanism. A new development was initiated in 1939 by Engelhardt and Lyubimova<sup>12, 13</sup> when they tested this idea by studying the interaction between ATP and myosin, which at that time, was the main protein considered to be associated with contraction. Under the stimulus of their observations, the reaction between ATP and the muscle proteins has been extensively studied and considerable progress has been achieved essentially by the work of the Needhams and Szent-Györgyi and their associates<sup>14, 15, 16</sup>. The demonstration by Straub of a second protein, actin, which combines with myosin to actomyosin, was an important advance in the study of the primary reactions which may underly the contractile process<sup>17</sup>.

However, if the interaction of ATP and actomyosin is studied in solution, the element of organization of the protein is not included. Recently, SZENT-GYÖRGYI\* has described a muscle fiber preparation which contracts in the presence of ATP. The usual electric stimulus is ineffective. This indicates that the conductive membrane is inactive. In a normal muscle fiber, whether stimulated directly or indirectly, the activation of the conductive membrane which envelopes the muscle cell intervenes between stimulus and contraction. It is only through the activity of this membrane that the contractile process is initiated. Since in the preparation of SZENT-GYÖRGYI the conductive mechanism is excluded but the contractile units are still functioning, as demonstrated by the ATP induced contraction, this fiber offers a most suitable material for the study of factors influencing contraction independent of the action of the conductive membrane. Such a differentiation is of considerable interest for the understanding of some muscular disorders, especially myotonia and familial periodic paralysis. It is with this problem in mind that the present study has been initiated.

# ${\bf MATERIAL}_{\underline{f}}^{\underline{f}}{\bf AND}_{\underline{f}}^{\underline{f}}{\bf METHODS}$

The psoas major of a rabbit was isolated by dissection and then tied at either end to an applicator stick. This preserved the resting length. The muscle was removed in toto by severing its connection at origin and insertion. It was placed in 50% glycerol, kept in the icebox overnight and then stored in 50% glycerol at —10° C. The fibers of the psoas muscle of the rabbit pass throughout the length of the muscle in a parallel fashion. For the present study this muscle appeared suitable but other striated muscles may be used in a similar way.

The main features of the glycerol preserved fibers are: the ease with which a small number of fibers (about 3-10) can be stripped from the main bulk of the muscle; the retention of the structural organization of the fibrils; the modification of the cell membrane to an unexcitable state; and finally the fiber's ability to contract on the addition of ATP.

By grasping the desired amount of muscle fibers in a forceps, they can be peeled from the muscle belly by exerting a slight tension. Forceful pulling on the fibers being detached causes partial interruptions in their continuity which can be noted by holding the fibers to the light and observing regions of increased transparency. Fiber groups 0.5 to 1 mm in diameter were separated from the muscle for study.

The microscopic appearance of the unstained preserved fibers was similar to the normal untreated fibers from the same animal. However, the volume of sarcoplasm was diminished and the diameters of the fibers were decreased appreciably.

The contractions of the unloaded fibers were studied in various experiments. After a number of preliminary observations, the experiments were carried out in the following way. The fibers were suspended in a constant volume of mammalian Ringer's solution according to Krebs. The contractions were recorded by an isotonic system on a kymograph moving at 3 cm per minute. The suspended fibers were kept in a bath of constant temperature which could be varied according to

 $<sup>^{\</sup>star}$  I am greatly indebted to Professor Szent-Györgyi for the demonstration of this preparation which made this study possible.

the requirements of the experiment. The standard ATP solution or others tested were added at a rapid and fairly constant rate reaching the suspended fiber almost instantaneously.

Electrical stimulation applied directly to the fibers did not cause contraction. The fibers were inert to supramaximal single and tetanic shocks. On the addition of ATP to the environment of the fibers, a definite and easily recorded contraction developed. As the fibers shortened, their diameters increased. In this respect the contractions resembled isotonic contractions of normal muscle. However, the fibers did not readily relax following the contraction induced by ATP. It was, therefore, necessary to use new fibers for each determination. The stability and constancy of fiber groups became all the more important for this reason. During the first two weeks of preservation the fibers were found to be unstable and variable. On exposure to isotonic solutions of salts, e.g., contained in mammalian RINGER's or saline, pseudo-contractile movements were occasionally observed. After the second week of preservation, more dependence could be placed on the stability of the fibers.

The ATPase activity of the homogenates of the fibers was determined by the method described

by Du Bois and Potter<sup>18</sup>.

## RESULTS

# Addition of ATP

Of the compounds tested, ATP and ADP alone elicited contraction of the fibers\*. A particularly significant group of substances are listed in Table I. The fiber apparently

TABLE I

compounds tested to induce contraction of unloaded non-conductive muscle fibers (rabbit). The  $\overline{ATP}$  and  $\overline{ADP}$  figures indicate the lowest concentration with which contraction was observed. The figures of the other compounds indicate the highest concentration tested

| Compound                                                                                  | Concentration (mg/ml) | Contraction |
|-------------------------------------------------------------------------------------------|-----------------------|-------------|
| Adenosinetriphosphate (ATP)<br>Adenosinediphosphate (ADP)<br>Adenosinemonophosphate (AMP) | 0.04<br>0.5<br>100.0  | +<br>+<br>o |
| Inorganic Pyrophosphate                                                                   | 44.0                  | O           |
| Acetylcholine                                                                             | 2.0                   | O           |
| Adrenaline                                                                                | 2.0                   | O           |

reacted in a selective manner to ATP and ADP. The threshold concentration of ATP requisite for contraction was less than that of ADP. Moreover, with equimolar solutions of ATP and ADP, the degree of shortening was greater in the case of the former. The amount of shortening of fibers was found to depend on the concentration of the ATP solution employed, approaching a maximum asymptotically (Figs 1, 2).

Unlike myosin threads, the loaded fibers contracted rather than extended in the presence of ATP. Moreover, if the fibers were incapable of shortening because the load was excessive, extension did not occur on the addition of ATP.

# Effect of temperature

When the suspended fibers and the added solutions of standard ATP were maintained at 37 °C, the extent of shortening was 5.4 times as great as observed under similar conditions at 10° C (Fig. 3). Calculated on this basis there was an increase in the amount of contraction by a factor of 1.9 for each 10° C rise in temperature between 10° and 37° C.

<sup>&</sup>lt;sup>\*</sup> I am greatly obliged to Dr Harry G. Albaum, Brooklyn College, for supplying adenosine-diphosphate and adenosine-monophosphate. The ADP was tested enzymatically by Dr Albaum and found by his method to be free of ATP.

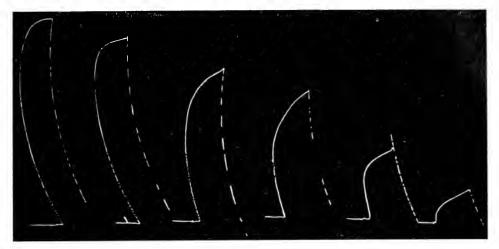


Fig. 1. The series of tracings represent the ATP induced isotonic contraction of fibers 8.5 cm in length recorded on a kymograph moving 3.0 cm per minute. Concentrations of ATP decreasing from 0.04 M in the first to 0.001 M in the last tracing. Magnification 6 ×.

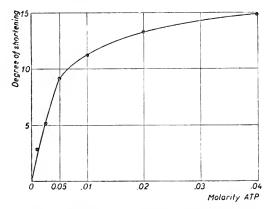


Fig. 2. Degree of shortening of fibers suspended in an isotonic recording system as function of increasing concentrations of ATP. Ordinates: Degree of shortening at a given time in arbitrary units. Abscissae: M ATP concentration. All fibers were of equal length (8.5 cm).

Fig. 3. Effect of temperature on the ATP induced isotonic contraction. Lower curve contraction at 10°C, upper curve at 37°C.

ATP concentration 0.002 M.

# Effect of $p_H$

The present experiments were carried out at  $p_{\rm H}$  7.4-7.6. It was observed that the fibers deteriorated rapidly in solutions beyond the limits of  $p_{\rm H}$  6.8 and 7.8.

# Effect of ions

Sodium ATP caused contraction of the fibers in the absence of other ions. However, magnesium ion activated the reaction of ATP with the contractile proteins of the fibers as shown by the increased extent of shortening in equimolar solutions of ATP (Fig. 4). The optimal concentration of magnesium ion was  $1 \cdot 10^{-2}$  M. Potassium in similar concentrations did not manifest the activating effect of magnesium. In the presence of References p. 67.

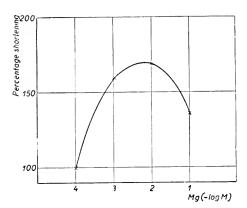


Fig. 4. The effect of magnesium ion in varying concentrations on the extent of isotonic shortening of fibers exposed to 0.002 M sodium ATP. The isotonic shortening caused by sodium ATP alone is arbitrarily assigned as 100%.

calcium ion at  $1 \cdot 10^{-2}$  M there was precipitation of the nucleotide and therefore the effect cannot be evaluated. On the basis of these observations a solution of ATP in  $1 \cdot 10^{-2}$  M MgCl<sub>2</sub> was used as standard to produce contraction.

# ATPase activity

The rôle of ATPase in the interaction between muscle protein and ATP has been repeatedly investigated. It is still a matter of discussion<sup>16, 19</sup> at which phase of muscle activity the enzyme is required. It appeared therefore of great interest to determine to what extent the ATPase activity is preserved in the preparation used. Table II shows the rate of decrease of the enzyme activity. The determinations revealed a gradual decline of

activity to about 20% of the initial value, at which level the activity appeared to remain stable.

On the addition of ATP enzymatically inactive fibers when loaded remained at resting length and no extension was noted.

TABLE II ATPASE ACTIVITY OF MUSCLE FIBERS OF RABBIT PRESERVED IN GLYCEROL AT — $10^{\circ}$  C, TESTED AT  $37^{\circ}$  C

| Time of preservation (days) | μg P/mg/15 min |  |
|-----------------------------|----------------|--|
| o (fresh                    | 25-30          |  |
| 4                           | 15-17          |  |
| 16                          | 10-12          |  |
| 20                          | 6-8            |  |
| 23                          | 6-8            |  |
| 30                          | 6-8            |  |
| 3-                          |                |  |
|                             |                |  |

# Inhibitors of contraction

Since it is known that ATPase has -SH groups<sup>13</sup>, the effect of -SH inhibitors were studied to find reversible inhibitors of the contractile process. Fiber bundles of a diameter of 0.5 mm or less were soaked in solutions of various compounds and then immersed in  $1 \cdot 10^{-2}$  M ATP standard. In suitable cases, the fibers after soaking were set up in the isotonic system and quantitative measurements made.

It was apparent that compounds which combined with sulfhydryl groups effectively inhibited contraction of the fibers (Table III). Of these compounds sodium  $\sigma$ -iodosobenzoate and mapharsen (meta-amino-para-hydroxyphenylarsinoxide) proved to be reversible inhibitors. The inhibitory effect of mapharsen was reversed by washing the fibers in saline whereas addition of cysteine to saline was required to remove the inhibition produced by  $\sigma$ -iodosobenzoate. HgCl<sub>2</sub> in  $\tau \cdot \tau o^{-4}$  M concentration caused irreversible

References p. 67.

TABLE III

INHIBITION OF ATP INDUCED CONTRACTION IN NON-CONDUCTING MUSCLE FIBERS (RABBIT) BY SOME COMPOUNDS REACTING WITH -SH GROUPS. AFTER EXPOSURE THE FIBERS WERE SOAKED IN SALINE CONTAINING O.OI M CYSTEINE EXCEPT IN THE CASE OF MAPHARSEN IN WHICH SALINE ALONE PROVED TO BE EFFECTIVE

| Compound          | Concentration (M) | Exposure (min) | Time of washing (min) | Reversibility |
|-------------------|-------------------|----------------|-----------------------|---------------|
| -Iodosobenzoate   | 1.10-3            | 20-25          | 120                   | +             |
| -Iodosobenzoate   | 5.10-1            | 30-40          | 90                    | +             |
| <b>I</b> apharsen | 1.10-1            | 20             | 10                    | +             |
| Iercuric chloride | 1.10-1            | 20             | > 120                 |               |
| Iercuric chloride | 1.10-3            | 9              | > 120                 |               |
| I,O,              | 3.10-1            | 7              | > 120                 |               |

inhibition of contraction. At a concentration of  $1 \cdot 10^{-5}$  M, however, the inhibitory effect of this compound appeared neglibible.

Other compounds tested and found without an inhibitory effect on the contractile process were sodium monoiodoacetate  $\mathbf{1}\cdot\mathbf{10^{-2}}$  M, sodium pyrophosphate  $4.4\cdot\mathbf{10^{-2}}$  M, sodium arsenate  $3\cdot\mathbf{10^{-2}}$  M, sodium arsenate  $1\cdot\mathbf{10^{-2}}$  M and antimony, tartrate and chloride,  $8\cdot\mathbf{10^{-2}}$  M.

Since o-iodosobenzoate is a reversible inhibitor of contraction, the following experiments were carried out. Thirty fiber units 6–8 cm in length were placed in a solution of o-iodosobenzoate  $5 \cdot 10^{-4}$  M in saline. At various intervals 2 cm were cut from some of these fibers, and the sections tested for contractility in a standard ATP solution  $1 \cdot 10^{-2}$  M. After 40 minutes, none of the parts of the fibers so tested contracted on exposure to the ATP standard. The fibers were then removed from the inhibiting solution. Ten were placed in saline containing cysteine in  $1 \cdot 10^{-2}$  M, the remainder in ATP  $1 \cdot 10^{-2}$  M for either 2 or 10 minutes. The experimental groups which did not contract during exposure to ATP were removed from the ATP and washed in saline for 10 minutes and then soaked in saline with  $1 \cdot 10^{-2}$  M cysteine for 12 hours. The fibers placed directly in the cysteine saline solution were tested by removing a unit and exposing it to ATP  $1 \cdot 10^{-2}$  M. Contractility had returned in 90 minutes. The fibers which were soaked either 2 or 10 minutes in ATP prior to their transfer into the cysteine saline were tested for return of contractility in a manner similar to the former group. During the 12 hours of observation, measurable shortening responses did not appear.

When fibers soaked in o-iodosobenzoate  $1\cdot 10^{-3}$  M ceased to contract, they were washed in saline for 10 minutes and homogenized. At that period, the ATPase activity of their homogenates ranged from 3–5  $\mu g$  P/mg/15 min. A part of the saline washed fibers were then regenerated in a solution of cysteine  $1\cdot 10^{-2}$  M. At the earliest moment when contractility returned, the homogenate revealed an ATPase activity of 5.5–8  $\mu g$  P/mg/15 min.

Fibers preserved in glycerol and then soaked in cold saline for 10 days retained their ability to contract when their ATPase activity was 6  $\mu$ g P/mg/15 min or above. Below this level contraction was absent.

# Effect of biologically active compounds

Fibers were exposed for 30 to 60 minutes to a number of substances known to have an effect on the contraction of normal muscle. In Table IV are listed the compounds *References p. 67*.

and the concentrations used. The degree of shortening on addition of standard ATP was compared with control fibers. To determine the possibility of simultaneous activation of the contractile process, test solutions of adrenaline, acetylcholine and histamine were prepared in ATP standard. These were added to fibers which had been previously soaked in corresponding solutions without ATP. None of the compounds enumerated affected the ATP induced contraction, whether the fibers were exposed to them prior to the contact with ATP or simultaneously.

TABLE IV

COMPOUNDS WHICH HAD NO EFFECT IN THE CONTRACTIONS INDICATED ON THE NON-CONDUCTING MUSCLE FIBER (RABBIT) NOR CHANGED THE ATP INDUCED ISOTONIC CONTRACTION.

TIME OF EXPOSURE: 30-60 MIN 24° C

| Compound                                                                 | Concentration<br>mg/mm                                        | Compound                                                                            | Concentration mgm/mm                       |
|--------------------------------------------------------------------------|---------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------|
| Adrenaline<br>Acetylcholine<br>Eserine<br>Prostigmine<br>Caffeine<br>DFP | 0.002-0.09<br>1.0 -2.0<br>0.05 -2.0<br>0.5 -1.0<br>0.5<br>1.0 | Strychnine<br>Veratrine<br>Ryanodin<br>Digitoxin<br>Histamine<br>Quinine<br>Cocaine | 0.5<br>0.5<br>1.0<br>0.2<br>1.0<br>0.4-0.6 |

## DISCUSSION

The SZENT-GYÖRGYI preparation may be considered a prototype of the contractile elements of normal muscle. For the study of contraction, it is intermediate between the intact cell and isolated systems (and proteins) in solution. Since the structure of the preserved fibers appears similar to the normal, they probably retain a considerable degree of the orientation and organization of the contractile proteins originally present. Partly for this reason, contraction rather than extension, as seen in the randomly constituted myosin threads, occurs after the addition of ATP to the loaded fibers. Also the supportive action of the sarcolemma mechanically prevents separation of the fibrils' contractile units while they are undergoing spatial rearrangement associated with the process of contraction.

ATP and ADP but not adenylic acid cause contraction of the fibers. Quantitative relationships between concentrations of ATP and ADP and the degree of shortening of the fibers require further investigations. It is apparent, however, that ATP is at least 10 times more effective in causing shortening than an equivalent amount of ADP. Since no enzyme is known to exist in muscle which splits ADP, the effect obtained with ADP may appear surprising. In previous observations reported ADP preparations were not entirely free of significant amounts of ATP and the action of such preparations could be attributed to ATP. The preparation of ADP used in these experiments was free of ATP, as tested enzymatically. However, it is possible that the ADP was converted by myokinase to ATP prior to its action. The fibers have not been examined for the presence of this enzyme.

Under the conditions of the present experiments, contraction of the fibers was not followed by comparable relaxation despite washings in solution containing NaCl, KCl, References p. 67.

CaCl<sub>2</sub> or MgCl<sub>2</sub> in various concentrations. Fibers which contracted as little as 20% of initial length were not restored to their original length. Relaxation may be a more complicated process than contraction depending on the integration of several reactions performed poorly, if at all, in this preparation. That ATP induces contraction and not relaxation of the fibers does not indicate at which phase of contraction dephosphorylation of ATP occurs<sup>19</sup>.

It has been observed that fibers inhibited from contraction by o-iodosobenzoate and then exposed for 2 minutes to ATP did not regain their contractility after prolonged washing in cysteine saline. This may indicate a reaction of ATP with proteins of the fiber possibly independent of that initiating contraction. This observation may offer an explanation for the inability of the fibers to relax, since in the usual experiments performed to measure isotonic contraction, the fibers were exposed to ATP for periods longer than 2 minutes.

It is noteworthy that the contraction of the fibers produced by ATP is enhanced by the addition of magnesium ions. This effect finds its analogy in the action of this ion in increasing the adsorption of ATP by actomyosin<sup>16</sup>. The magnitude of the effect and the optimal concentration of magnesium ion at which it occurs are in harmony with similar observations in isolated ATP-actomyosin systems.

Activation of the fiber contraction by magnesium contrasts to its depressing effect on the intact muscle<sup>20</sup>. Further observations are necessary to decide whether this may indicate that the magnesium effect in the intact fiber is due to an action on the conductive membrane.

Compounds like mapharsen and o-iodosobenzoate which reversibly inhibited contraction of the fibers inactivate ATPase activity of myosin²¹. The inhibitors are not specific for ATPase but rather oxidize or combine with thiol groups in general. By measuring the ATPase activity of the homogenates of the fibers, one may secure an index of their efficacy in affecting available –SH groupings. However, the inactivation of ATPase may not be directly correlated with the ability of these inhibitors to prevent contraction. The sulfhydryl groups binding actin to myosin, e.g., are susceptible to effects of these inhibitors²¹. The loss of fiber contractility may be related to a stabilization or blocking of sulfhydryl linkages of the contractile proteins themselves.

By means of the elemental contractile system under study, the action of the biologically important compounds listed in Table IV can be further differentiated. All the substances enumerated are known to affect the process of contraction of intact muscle fibers. Since they are ineffective in influencing fiber contraction produced by ATP, their site of action may be assumed to be elsewhere. From data available it is probable that they affect contraction of intact fibers through their action on the conductive membrane of the muscle either at the neuromuscular junction or along the fibers. Of particular interest in this connection is the absence of any effect of the cholinesterase inhibiting compounds, such as diisopropylfluorophosphate and eserine, on the contractile process. This does not support the assumption of a general toxic effect of these compounds as proposed by some investigators, but is consistent with the view which attributes their effect to blocking conduction<sup>22</sup>.

The observations presented show the usefulness of the non-conductive contractile preparation of muscle described by SZENT-GYÖRGYI. The system simplifies the study of the contractile process and offers an opportunity to study chemical and pharmacological factors affecting contraction as distinct from conduction.

I am grateful to Dr David Nachmansohn for his suggestions and advice in the conduct of this research.

#### SUMMARY

A preparation of muscle fibers preserved in glycerol has been described by SZENT-GYÖRGYI, in which the contractile elements remain intact whereas the conductive membrane is not functioning. Properties of such fibers and factors influencing the contractile mechanism independent of conduction have been studied. The following essential results have been obtained.

1. Of a great number of compounds tested, only ATP and ADP induced contraction. The concentration of ADP required was more than ten times higher than that of ATP. Adenylic acid and inorganic pyrophosphate had no effect in high concentrations. The same is true for a great number of compounds like acetylcholine, adrenaline, DFP, eserine and many others which are known to affect the normal muscle fiber preparation.

2. Quantitative evaluations have shown that  $4\cdot 10^{-2}\,\mathrm{M}$  of ATP is close to the optimum to induce the contraction of the non-conducting fiber but concentrations as low as  $1\cdot 10^{-3}\,\mathrm{M}$  had a measurable effect

3. The extent of shortening increased strongly with temperature, for each  $10^{\circ}$  C rise between  $10^{\circ}$  and  $37^{\circ}$  C by a factor of 1.9.

4. The p<sub>H</sub> optimum was found to be between 7.4 and 7.6. The fibers deteriorated rapidly in solutions beyond the limits of 6.8 and 7.8.

5. Magnesium ions activate the reaction of ATP with the contractile proteins. The optimal concentration was  $1 \cdot 10^{-2}$  M.

6. The ATPase activity in the fiber preparation declined greatly during the first three weeks to about 20% of the initial value at which level the activity appears to remain stable.

7. The effect of –SH inhibitors has been studied. Two of these compounds, o-iodosobenzoate and mapharsen, proved to be reversible inhibitors of the contractile process.

#### RÉSUMÉ

SZENT-GYÖRGYI a décrit une préparation de fibres musculaires préservées dans le glycérol dans laquelle les éléments contractiles restent intacts tandis que la membrane conductive ne fonctionne pas. Les propriétés de telles fibres et les facteurs qui influencent le mécanisme contractile indépendant de conduction ont été étudiés. Voici les principaux résultats obtenus.

1. Sur un grand nombre de composés étudiés seuls l'ATP et l'ADP induisaient une contraction. La concentration d'ADP requise était plus de dix fois supérieure à celle d'ATP. L'acide adénylique et le pyrophospate inorganique n'avaient pas d'effect à des concentrations élevées. Il en était de même pour un grand nombre de composés tels que l'acétylcholine, l'adrénaline, le FDP, l'ésérine et beaucoup d'autres dont nous savons qu'ils affectent une préparation normale de fibres musculaires.

2. Des évaluations quantitatives nous ont montré qu'une concentration de 4·10<sup>-2</sup> M d'ATP est près de l'optimum qui induit la contraction d'une fibre non-conductive; cependant des concentrations aussi faibles que 1·10<sup>-3</sup> M produisaient un effet mesurable.

3. Le raccourcissement devenait plus fort lorsque la température augmentait; le facteur était de 1.9 pour toute augmentation de 10° C, dans l'intervalle de 10 et 37° C.

4. Le  $p_H$  optimum se trouvait entre 7.4 et 7.6. Le fibres se gâtaient rapidement dans des solutions ayant un  $p_H$  inférieur à 6.8 ou supérieur à 7.8.

5. Les ions de magnésium activaient la réaction de l'ATP avec les protéines contractiles. La concentration optima était de  $1\cdot 10^{-2}$  M.

6. L'activité adénosine triphosphatasique diminuait rapidement dans la préparation de fibres jusqu'à environ 20% de sa valeur initiale puis, à ce niveau, elle semblait rester stable.

7. Nous avons étudié également l'effet des inhibiteurs d'-SH; deux de ces composés, l'o-iodosobenzoate et le mapharsène sont des inhibiteurs réversibles du processus contractile.

#### ZUSAMMENFASSUNG

SZENT-GYÖRGYI hat ein in Glycerin konserviertes Muskelpräparat beschrieben, in dem die kontraktilen Elemente intakt bleiben, während die leitende Membrane nicht funktioniert.

Die Eigenschaften solcher Fasern und die Faktoren, welche den von Konduktion unabhängigen Kontraktionsmechanismus beeinflussen, wurden untersucht. Dies sind die wichtigsten Ergebnisse.

1. Von der grossen Anzahl der untersuchten Verbindungen bewirkten nur ATP und ADP eine Kontraktion. Die nötige Konzentration war für ADP zehnmal grösser als für ATP. Adenylsäure und anorganisches Pyrophosphat hatten in hohen Konzentrationen keine Wirkung. Das Gleiche gilt für

eine grosse Anzahl von Verbindungen, wie Acetylcholin, Adrenalin, DFP, Eserin und viele andere. deren Wirkung auf normale Muskelfaserpräparate bekannt ist.

2. Quantitative Schätzungen haben ergeben, dass das Optimum für die Kontraktion einer nicht leitenden Faser nahe bei 4·10-2 M ATP liegt, aber schon Konzentrationen von 1·10-3 M hatten eine messbare Wirkung.

3. Die Verkürzung wird bei steigender Temperatur grösser; für eine Steigerung von je 10° C

zwischen 10 und 37°C beträgt der Faktor 1.9.

4. Wir fanden ein pH-Optimum zwischen 7.4 und 7.6. Die Fasern verderben rasch in Lösungen deren pH unter 6.8 oder über 7.8 liegt.

5. Magnesiumionen aktivieren die Reaktion von ATP mit Kontraktions-Proteinen. Die optimale

Konzentration betrug 1 · 10<sup>-2</sup> M.

- 6. Die ATPase-Aktivität des Faserpräparates nimmt während der ersten drei Wochen stark ab und scheint dann bei ungefähr 20% des Anfangwertes konstant zu bleiben.
- 7. Die Wirkung von -SH-Hemmstoffen wurde untersucht und gefunden, dass zwei von Ihnen, Jodosobenzoat und Mapharsen reversible Inhibitoren des Kontraktionsprozesses darstellen.

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## PART II

# NERVE

# MORPHOLOGY IN MUSCLE AND NERVE PHYSIOLOGY

by

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As applied to biology, morphology embraces the study of the structure of cell and tissue constituents from gross and microscopic anatomy through the colloidal range and even to the molecular and atomic levels. With the introduction of electron microscopy it is now possible to visualize directly the structure of objects throughout the colloidal range. It is not unrealistic to expect that technical development will make possible direct visualization of such biologically important objects as the smaller protein molecules and possibly even the polypeptide chains. Simultaneously the theory and techniques of X-ray diffraction are also progressing. This method is already able to deal effectively with the analysis of the internal architecture of certain crystalline proteins; a major hurdle appears to be the development of suitable computing methods—a matter chiefly of technology and patience. Progress is also being made in the analysis of the less regularly constructed, but no less important biologically, fibrous proteins and complexes of proteins with lipids, nucleic acids and polysaccharides. This, too, is a matter of painstaking, patient development of techniques, experimental and theoretical.

Morphology is a science in its own right. The analysis of the detailed structure of the molecules and complexes which occur in tissues is largely the task of the physicist who, in turn, must depend upon the chemist to identify, isolate, purify and characterize the individual constituents. In the normal course, as physicists and chemists become interested in such substances, one may expect knowledge in this branch of crystallography slowly to unfold. Slowly because such complex, frequently imperfectly structured materials are not attractive to most crystallographers who are likely to regard them as "sick crystals", as one colleague expresses it. Actually, some of the most important protein crystals are far from "sick" structurally; upon the regularity of the internal structure of their molecules depend such fundamental vital properties as are manifested in the phenomena of immunology, genetics, and the ordered processes of growth and development. Relatively minute changes in the structure of certain protein molecules may make the organism sick (Pauling et al.<sup>1</sup>, recently referred to sickle cell anemia as a "molecular disease"!). The great biological significance of structural studies has stimulated many physicists and chemists to devote their efforts to the problem. Hopefully their numbers will grow.

The detailed analysis of biomolecular structure is a long term task. The analysis starts with a rough characterization of the main structural features of a particular tissue entity. With the aid of the electron microscope the biologist relatively untrained in the discipline of crystallography can and must take an active in this phase. As the analysis

becomes more detailed, eventually leading to the localization of the constituent atoms, the task becomes more that of the crystallographer. The physiologist and biochemist must make use of the information available at the moment in attempting to account for biological phenomena.

To what extent has structure analysis been of assistance in solving major physiological problems and what is the outlook for further advance in this field? In seeking a perspective regarding such a question a consideration of muscle contraction and nerve conduction may be instructive because of the contrast which these problems present in respect of inherent susceptibility to morphological investigation and to progress already accomplished. The following account is necessarily brief and attempts merely to indicate the trend of research in this field.

## MUSCLE CONTRACTION

Contractility is particularly favourable for morphological study because it involves structural changes at all levels of observation. The voluminous literature of muscle histology, devoted largely to striated muscle, led to few important physiological clues. Perhaps the "reversal of striation" on contraction was among the most suggestive. Even observations in polarized light were difficult to interpret. The positive form birefringence indicated that the fibrous proteins have widths small with respect to the wavelength of light. The relative isotropy of the I bands was long misinterpreted as indicating disorientation in these regions. Muralt and Edsall's demonstration of the positive birefringence of myosin focused attention on this protein as the contractile substance of muscle. Astbury's identification of myosin as the source of the wideangle X-ray pattern of muscle, together with his hypothesis of intramolecular folding during contraction, helped to seek in myosin the substratum of contraction³.

In the short time since electron microscopy has been applied to the problem, important advances have been made. The view that myosin is localized in the A bands, already discredited by quantitative considerations, was disproven by electron microscopy, which showed that the protein filaments extend as parallel bundles continuously through both A and I bands<sup>4</sup>. The relative isotropy of the I bands is therefore not due to disorientation. Recently the view has been taken that the isotropy results from the presence of negatively birefringent substances in the I bands which compensate the positive birefringence of the myosin; this material has been variously reported as nucleotides<sup>5, 6</sup>, lipids<sup>7</sup> and phosphoproteins (N material)<sup>8</sup>.

In contraction the protein filaments remain relatively straight and parallel, indicating that the contractile unit is thinner than the filaments (ca 150 Å). The distribution of the dense material in the A bands and on the Z membrane changes in agreement with the histological picture of reversal of striation.

Morphological studies were greatly stimulated by advances in our concepts of mechano-chemical coupling mediated by high-energy phosphate bonds and by the discovery by the Szeged group that myosin is composed of two proteins, a water-soluble myosin and actin, the actomyosin complex being sensitive to the action of adenosine-triphosphate (ATP). The general morphological features of the water-soluble myosin and the globular and fibrous actin were soon demonstrated with the electron microscope<sup>9</sup>, together with the dissociating effect of ATP on the actomyosin threads<sup>10</sup>.

Of great significance in the morphological approach to the contractile mechanism References p. 76/77.

is the axial periodicity demonstrated both by small-angle X-ray diffraction<sup>11</sup> and by electron microscopy<sup>4</sup>. This period has a value of about 400 Å in uncontracted fibres and appears to be characteristic of muscle generally, for Bear has observed it not only in vertebrate striated muscle but also in various invertebrate muscles which are generally regarded as being of the smooth type. In electron micrographs the filaments have a beaded appearance which gives rise to a fine banding of the myofibril, the distance between bands being about 400 Å. Draper and Hodge<sup>12</sup> have shown the period very strikingly in electron micrographs of platinum-shadowed preparations. In their preliminary note they state that the axial period varies inversely with the degree of shortening of the muscle. Variations in the 400 Å period with fibre length were also noted by Bennett<sup>13</sup> who believes to have shown that the filaments have a helical structure. If these points are satisfactorily documented and confirmed we shall have visual evidence of the contractile phenomenon at the near-molecular level.

Actually the relation between the 400 Å axial period demonstrated by X-ray diffraction and the pseudo-period of about the same value seen in electron micrographs is not clear. The largest meridional spacing observed in the X-ray patterns is about 27 Å which is an order of the larger period. If the situation is similar to that of paramyosin<sup>14, 15</sup> one might expect that the period which might be observable as cross bands in the electron microscope, would have a value of about 27 Å; the larger period of about 400 Å would be manifested as a geometric pattern of discontinuities within the bands. However, depending on the type of geometry of the intraperiod structure, discontinuities at a spacing larger than 27 Å may appear in electron micrographs. The solution of this problem will have to await a more detailed X-ray analysis and attainment of very considerably increased electron microscope resolution of the structure of the filaments.

ASTBURY, PERRY, REED, AND SPARK<sup>16</sup> have observed a spacing of 54 Å in fibrous actin. At large angles the pattern is not that of an alpha protein. This led the authors to the conclusion that the large-angle pattern of muscle is due to myosin while the small-angle pattern is due to actin; the full muscle pattern derives from actomyosin which exists as a complex in muscle. While this may prove to be the case, the diffraction evidence is not yet sufficiently detailed to require this conclusion.

The electron microscope investigation of contractility might be facilitated by examination of *in vitro* models such as the actomyosin-ATP system described by SZENT-GYÖRGYI<sup>17</sup>. This would be true if such systems permitted higher resolution than could be achieved in the myofibril and, particularly, if the essential properties of such a system faithfully portray those of muscle. Recently SZENT-GYÖRGYI<sup>18</sup> has found that muscles thoroughly extracted with 50% glycerol at low temperatures are capable of contraction when treated with ATP and produce the same tension as the intact muscle when maximally excited. Differences in the behaviour of this model as compared with intact muscle are attributed to the fact that the model may lack some of the proteins, lipids and other substances with which the actomyosin is normally associated in muscle. From studies of this model, as from the previous one of VARGA<sup>19</sup>, the conclusion was reached that contractile substance is composed of functional units, "autones", and that contraction represents an all-or-none equilibrium reaction of these units; contraction and relaxation are two distinct allotropic states of the autones.

Unfortunately, as admitted by SZENT-GYÖRGYI<sup>18</sup> and as amplified by SANDOW<sup>20</sup> none of the partial systems and models thus far proposed fully displays the essential *References p.* 76/77.

properties of muscle. So far as the morphological evidence is concerned, Perry, Reed, Astbury, and Spark<sup>21</sup> have shown by X-ray and electron microscope studies that the changes which occur when ATP is added to actomyosin is an intermolecular syneresis, the contraction occurring in a direction normal to that which characterizes muscle contraction. Moreover, there is no evidence from X-ray results for the existence of two distinct states of the "auxones". Upon contraction the large-angle pattern indicates a change from an alpha to a poorly defined, disoriented beta configuration. Efforts to obtain a characteristic small-angle pattern from contracted muscle have thus far met with failure. What little electron microscope evidence bears on this point suggests that the 400 Å axial period shows a continuous change in value with change in fibre length rather than two distinct states.

However valuable partial systems and models may be from the biochemical viewpoint, it is evident that, in the investigation of structural mechanism which is characteristic of muscle, final answers will be obtained by observation of nothing less complex than the muscle fibre itself.

There is no reason to doubt that the combination of X-ray diffraction and electron microscopy will be equal to the task of disclosing the molecular changes which occur in contraction. The small-angle X-ray analysis is particularly promising and may be expected in the near future to portray the main features of the lattice of Bear's Type II protein. The more detailed structure at smaller separations, involving the configurations of polypeptide chains in relaxed and contracted muscle seems more difficult of unique solution unless more diffraction data can be obtained at large angles.

An electron microscope investigation of the extra-filamentous structures of the striated myofibril, including the materials concerned in the "reversal of striation", the Z membranes and the binding material which connects filaments to each other and to the sarcolemma laterally, offers much promise. However, primary interest attaches to the detailed structure within the filament and the changes of this structure with contraction. As compared with paramyosin the task of the electron microscopist will be considerably more exacting because of the smaller spacings involved. Obviously, at this level of size the most critical judgement of image quality and of optical artifacts will be required.

## NERVE CONDUCTION

The problem of nerve conduction contrasts strikingly with that of muscle contraction as regards the contributions of morphology. This is due to the fact that the changes whicht occur in a nerve fibre when an impulse is conducted are far more subtle than those occurring during contraction and also to the fact that chemical characterization of nerve fibre constituents, particularly the proteins, is almost completely lacking. Until about the turn of the century the extensive histological literature emphasized primarily the neurofibrils which were regarded by many as the substratum of impulse conduction. In its most stimulating form this hypothesis visualized the interface between axoplasm and neurofibril as the locus of the electro-chemical changes which underlie impulse propagation<sup>22</sup>. Bethe's<sup>23</sup> demonstration of a difference of stainability of neurofibrils under the anode and cathode of a polarizing current, due to the presence in axoplasm of a hypothetical "fibrillary acid", attracted little attention though the phenomenon seems quite genuine and has some renewed interest in the light of recent

polarization experiments<sup>2a</sup>. The ascendency of the membrane theory together with a growing distrust of structures which can be demonstrated only after fixation caused physiologists to lose interest in morphology as an immediate aid in studying the mechanism of impulse propagation. To many physiologists the nerve fibre became essentially a tube limited by a metastable interfacial film and containing a salt solution plus certain metabolizing substances capable, in some way, of maintaining the structural integrity of the fibre and of furnishing the energy needed for impulse propagation.

The conservative nature of the processes involved in the generation and propagation of the spike wave was demonstrated by studies of the thermal and oxidative changes. The excess oxygen consumption per impulse may be very small at low rates of stimulation<sup>25</sup> and, after treatment with azide, nerve is capable of conducting action waves of undiminished amplitude with no accompanying increase in oxygen consumption<sup>26</sup>.

Currently there is renewed interest in the coupling of reactions of chemical metabolism with bioelectric processes. In addition to the much debated question of the role of acetylcholine<sup>27–29</sup> and of other "Erregungsstoffe" suggestions have been offered linking particular chemical reactions with the polarization potential<sup>31</sup>. ATP-ase has also been invoked<sup>32–34</sup>. However, there is as yet no general agreement as to the role of such substances.

In the field of electrophysiology much progress has been made in the more accurate description of the electrical properties of the nerve fibre at rest and during activity. However, the present period is characterized by fundamental disagreement among the most competent investigators about the nature, origin and significance of the polarization and action potentials<sup>35–37</sup>. Characteristic also is the failure of the electrical studies to provide definitive clues as to the structure and chemical composition of the reacting system.

The appalling ignorance about the chemical composition, particularly of the proteins, of peripheral nerve may in part be due to the unattractiveness of investigating a tissue in which the structure of interest is presumably a paucimolecular layer of uncertain location. Amino acid analyses have been made on the socalled "neurokeratin" but the location of this protein is uncertain. Originally the term was applied to the protein of the myelin sheath. However, BLOCK<sup>38</sup> concluded that it is more probably located in the axis cylinder and may be the protein of which the neurofibrils are composed. A pseudo-nucleoprotein was isolated from the axons of the giant fibres of the squid and from lobster nerves<sup>39</sup>. Since this complex seems to occur in the central nervous system as well as in peripheral nerve it was considered characteristic of nerve and was termed "neuronin". Its possible relation to neurofibrils is not known. The chemical characterization of this entity is at best very sketchy, but it can at least be definitely localized in the axon. Chemical investigations are now being carried on by J. Folch and his collaborators on the proteins and lipids of the brain. Already a liponucleoprotein and several other proteins have been isolated and partially characterized<sup>40</sup>. Though it is impossible at present to say whether these proteins are located in the perikarion, the axon or in extrafibrillar material, it may be possible, once the pure constituents are thoroughly characterized, to devise methods by which their presence in the components of peripheral nerve may be demonstrated.

In view of the situation as outlined above, it is perhaps not surprising that morphological studies have thus far contributed relatively little to an understanding of References p. 76/77.

impulse propagation in nerve. To gain a perspective as to the promise of further investigations at or near the molecular level it will be useful to consider what information of this sort is now at hand. The discussion will be centered around the two chief components of the fibre, the axon (myelin) sheath and the axon (axis cylinder).

## THE AXON SHEATH

The general features of the molecular architecture of the myelin sheath have been deduced from polarized light and X-ray diffraction studies<sup>41</sup>. Essentially the sheath consists of lipid-protein layers about 180 Å thick wrapped concentrically about the axon. The lipid phases exist as smectic mesomorphic double layers of mixed lipids, the paraffin chains being oriented normal to the planes of the layers, i.e., radially in the sheath. The protein component is intercalated between double layers of lipids in thin sheets estimated to have an over-all thickness of 25–30 Å per period. This is presumably the protein which, on fixation, gives rise to the neurokeratin network. In view of our ignorance of the properties of this protein it is impossible to say anything about its configuration in the very thin layers in the sheath. When nerve is dried the thickness of the layers is reduced by about 25 Å and a considerable fraction of the sheath lipids is extravasated from the organized structure to form separate lipid phases. In the skeleton of the original structure which remains it seems probable that a fraction of the lipid molecules is firmly bonded to the thin protein layers and that this linkage maintains the structure in the dried sheath. The nature of this linkage can only be surmised though one may suspect that the acid groups of the cephalin molecules may be involved.

Thus far electron microscopy has contributed little to our knowledge of sheath structure though advances in this direction may be expected when sectioning methods are applied. From osmic acid fixed nerves disintegrated with sonic oscillations, SJÖSTRAND (unpublished) has observed fragments of very thin layers which may have been derived from the myelin sheath. He had previously demonstrated with the electron microscope that the outer limbs of the retinal rods consist of stacks of thin discs42, 43. This is in agreement with the polarized light analysis which indicated that, like those of the myelin sheath, the thin layers contain lipid and protein components oriented perpendicular and parallel, respectively, to the planes of the layers. It has been suggested<sup>44</sup> on very inadequate grounds, that the protein of the rod outer limbs may be a type of "neurokeratin". DE ROBERTIS and the writer have also observed thin layers in preparations from fragmented myelinated nerves. Curiously the fragmented layers frequently show characteristic angular cleavage. If the layers actually derive from the sheath this type of cleavage is unexpected since the sheath has thus far been considered to be uniaxial with optic axes normal to the layers. Measurements of the thickness of the layered fragments may help determine their origin since the over-all thickness of the sheath layers is known from X-ray data.

The X-ray and polarized light results concern only the highly organized lipid-protein substance of the sheath. Determination of the detailed structure of the various other sheath components which have been observed histologically must await electron microscope study in thin sections. Among these structures are the boundaries of the sheath at the incisures, the Golgi funnels and spirals of Rezzonico, the axolemma membrane, the Schwann cell and the outer fibrous investiments. The structure at the node will be particularly interesting because the limiting envelope of the fibre at this point

has especial physiological significance. Technical difficulties make it hard to study this surface structure with polarized light.

From polarized light studies it has been suggested that all nerve fibres may possess a lipid-protein sheath having the same type of architecture as that of the myelin sheath <sup>45</sup>. Such a sheath has been demonstrated in several types of invertebrate fibres though the investigation has not yet been extended to the so-called naked fibres such as the Remak fibres. In the limiting case the naked fibre may possess a surface structure no more complex than the plasma membrane itself. The polarized light method is probably sufficiently sensitive to detect molecular orientation in such paucimolecular layers. However, the bearing of such data on the problem of impulse propagation would still remain to be shown.

No direct connection between sheath ultrastructure and physiological properties has been demonstrated, although a correlation has been pointed out between sheath birefringence, e.g., essentially lipid concentration, and velocity of impulse propagation<sup>45</sup>. This correlation is at best only rough when applied to the fibres of a particular type of nerve but seem more suggestive when fibres of widely different types of nerves are considered. For several types of vertebrate and invertebrate fibres having approximately equal conduction velocities, Taylor<sup>46</sup> found that the product of fibre diameter and sheath birefringence is roughly constant.

#### THE AXON

The most interesting structures in the axon are, of course, the neurofibrils. Only in exceptional cases can these objects be observed in the fresh fibres, the chief lore of the literature being concerned with fixed and stained preparations. The neurofibrils may approach the limit of microscopic resolution in fixed and stained preparations. Hence it is readily understandable that, if they pre-exist in the fresh axon, they may not be visible, particularly if refractive index relations are unfavourable. In the dark field microscope Ettisch and Jochims<sup>47</sup> observed no structure in the fresh axon though very fine collagen fibrils of the connective tissue were clearly visible, indicating a fundamental difference in the two types of fibres. After treatment with reagents such as CaCl<sub>2</sub> or fixatives, neurofibrils immediately appear. Apparently only slight colloidal alterations suffice to make them visible. It was concluded by Peterfi<sup>22</sup> that the fresh axon is a rodlet sol capable, under very slight chemical provocation, of forming a fibrous system. He suggested that the mutual interaction of the elongated micelles may be intimately associated with impulse propagation.

Electrical studies have failed to indicate any direct role of axoplasm except as a passive conductor of current. An electrode may be inserted into the axon of the squid giant fibre without blocking conduction. But if the inner surface of the cell membrane is injured conduction ceases<sup>48, 49</sup>. However, Curtis and Cole's<sup>49</sup> statement that "This makes it seem rather unlikely that there is an internal structure in the axon which takes a prominent part in the active mechanism of propagation" must be accepted with caution since there is no evidence that the manipulation mentioned disrupted any axonic structures which might be present as it did the membrane structure.

Changes in the colloidal organization of the axon with activity have been sought, but thus far the experimental techniques have been very crude. It has been claimed that the fibre exhibits changes in contour with electrical polarization, swelling at the

anode and flattening at the cathode<sup>22</sup>. More recently Flag<sup>50</sup> believed to have shown that the viscosity and turgor of the axoplasm of the squid giant fibre is considerably increased during activity. He suggested that excitation increases the viscosity by shifting the sol-gel equilibrium. If Flag's results are confirmed, careful investigation of the light scattering by the axon might be warranted. The existence of elongate particles in the fresh axon is demonstrated by the positive birefringence which, though weak, is measureable in large axons such as in the squid giant fibre. Semi-quantitative analysis of the positive form birefringence indicated that though the oriented fibrous structures occupy a small portion of the axon volume, they must have a considerable degree of regularity of internal structure, for their intrinsic birefringence is comparable with that of myosin or collagen fibres<sup>51</sup>.

No change in molecular orientation in the axoplasm of squid giant fibres during activity could be detected by polarization optical means<sup>52</sup>. Using a sensitive photoelectric method capable of recording small changes in birefringence without appreciable time lag, it was concluded that if any change occurred it was less than 0.2% of the initial birefringence for the spike process and less than 0.08% for the slow recovery processes. Unless more sensitive methods yield positive results it may be concluded that impulse propagation is associated with little if any change in orientation of the elongate particles of the axon.

From electron microscope studies, RICHARDS, STEINBACH, AND ANDERSON<sup>53</sup> described contorted fibrils composed of kinked elongate particles in axoplasm extruded from squid giant fibres. They suggested that these structures may form the basis of neurofibrils. DE ROBERTIS AND SCHMITT<sup>54</sup> observed characteristically double-edged fibrils in electron micrographs of material obtained by sonic fragmentation of frozen sections of formalin fixed nerves of various types. Such structures had never before been observed. For descriptive purpose the fibrils were tentatively called "neurotubules". The dense material at the edges is for the most part removed by washing with water. It is not yet clear to what extent this dense material is associated with the fibrils in the natural state and to what extent it may have been deposited upon them during the preparative procedure.

After staining with phosphotungstic acid or shadowing with heavy metal the fibrils have a cross-striated appearance. The axial period averages about 650 A and detailed intraperiod structure has been observed. Since this period is similar to that of collagen<sup>55</sup> and since nerve fibres are closely invested with connective tissue the possibility that neurotubules may be collagen fibres invested with dense materials of undetermined origin was carefully considered. The fragmentation technique employed makes it difficult to determine the location of the neurotubules in the nerve fibre. All the evidence was consistent with the view that they are of axonic origin. Important in the reasoning was the fact that typical double-edged fibrils were not observed in preparations of nerves which had been allowed to undergo degeneration in vivo (Wallerian) or in vitro<sup>56</sup>. However, in recent experiments on late degeneration, results at variance with those previously described were obtained. Preparations from nerves degenerated for as long as three weeks were not devoid of double-edged fibrils but contained them in considerable abundance. The reason for this discrepancy is not clear. However, in view of the importance of the degeneration changes to the argument that fibrils are of axonic origin, the entire matter is being reinvestigated. Speculation as to the possible role of the neurotubules in nerve function would be premature at this time.

Recent experiments suggesting that axoplasm may be continuously moving peripherally from the cell body in the normal neuron<sup>57, 58</sup> have stimulated renewed interest in the colloidal properties of the axon as they concern trophic phenomena. It seems probable that application of the thin sectioning technique may prove valuable in studying axon structure with the electron microscope and that such studies may throw light on the physical basis of trophic processes.

The axons of fresh fibres offer little promise for X-ray diffraction studies because of their high water content. It was estimated that the axon proteins of the squid giant fibre account for only 3 or 4% of the wet weight of the fibre<sup>51</sup>. Dried frog, lobster and crab nerves show equatorial diffractions at about II Å. It is probable that these diffractions arise from connective tissue because alcohol-dehydrated axons isolated from squid giant fibres showed only two disoriented rings at about 4.7 and IO Å, characteristic of denatured protein<sup>59</sup>. These patterns are similar to those obtained from fibres spun from axis cylinder protein. These X-ray investigations of axon structure were not exhaustive and, in view of current electron microscope results, warrant further careful study.

From the above account it is clear that the problem of structure analysis in nerve is a formidable one. It is particularly challenging because of the high sensitivity of the colloidal organization to physical or chemical manipulation and because the chemical reactions underlying the physiological process are completely unknown.

There can be little doubt that X-ray and electron microscope techniques, if sufficiently acutely applied, are capable of penetrating to or near the molecular level in nerve as has already been accomplished in the case of contractile tissue. Hardly more than a beginning has been made thus far. Progress with the morphological problem would be greatly accelerated if the chemical properties of the nerve proteins were known. The biochemical problem is itself quite formidable bur there is no reason to doubt that it would yield if subjected to a concerted attack by modern methods of isolation and characterization. The bioelectric aspects have attracted the best efforts of many competent investigators and their analysis is still proceeding. The time has come for an equally concentrated attack upon the morphological, biochemical and enzymological aspects. Only thus may we expect to make significant progress with a problem as complex as that of nerve function.

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# STUDIES ON PERMEABILITY IN RELATION TO NERVE FUNCTION

# I. AXONAL CONDUCTION AND SYNAPTIC TRANSMISSION

by

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## INTRODUCTION

Cellular boundaries are endowed with the ability either to permit or to prevent the entrance and leakage of various compounds and metabolites. This makes possible the elimination of waste products and the supply of substances important for ionic equilibrium, energy requirements, and other vital functions of the cell. There are many indirect indications for the selective permeability of the membranes covering the cell. The great importance of this property for the understanding of cellular mechanisms and of the action of compounds applied externally, which includes most pharmacological effects, has long been recognized. Nevertheless, surprisingly little is known in regard to the factors which determine and affect permeability of cellular boundaries. Direct measurements are extremely difficult. The introduction of isotopes as research tool in biology, mainly due to the work of Hevesy¹ and Schoenheimer and Rittenberg³, has opened a new pathway to the approach of the problem, but the obstacles to be overcome are still tremendous. The lucid appraisal of the field by Kroch³ in his Croonian lecture shows that in spite of some progress in recent years this aspect of cellular function is in its initial phase.

The permeability of the surface membranes of the nerve cell is of particular interest. Physiologists of the last century have already postulated that changes in permeability must be intimately associated with the function of the neuron, *i.e.*, with the propagation of the nerve impulse. Du Bois-Reymond who first established conclusively that nerve activity is associated with flow of current devoted much time to testing the possibility that the source of the electromotive force for the electrical manifestations observed may be ionic concentration gradients between the interior of the cell and its outer environment. When, in the later part of the nineteenth century, physico-chemical investigations revealed the marked potential differences which may be produced by semipermeable membranes, the existence of such membranes was postulated as a basis for the electrical manifestations during the passage of the nerve impulse. Ostwald wrote in 1890: "An den halbdurchlässigen Membranen kommen weit grössere Potentialdifferenzen zustande als in gewöhnlichen Flüssigkeitsketten. Es ist vielleicht nicht zu gewagt schon hier die Vermutung auszusprechen, dass nicht nur die Ströme in Muskeln und Nerven sondern auch namentlich die rätselhaften Wirkungen der elektrischen Fische durch die hier

erörterten Eigenschaften der halbdurchlässigen Membranen ihre Erklärung finden werden". From the discussions of Du Bois-Reymond, Hermann, Ostwald and others concerning the mechanism underlying the generation of the electric currents during nerve activity there finally emerged the membrane theory formulated by Bernstein early in this century. This theory forms the basis of all modern concepts of conduction and has been an extremely useful working hypothesis. Essentially the theory assumes that the nerve fibre in resting condition is surrounded by a polarized membrane, selectively permeable to potassium ions. The concentration of these ions inside the nerve fibre is high compared with that outside. There is, therefore, a tendency for the potassium ions to move to the outside, but they are kept back by the negative ion for which the membrane is impervious at rest. There thus develops a positive charge on the outside surface of the membrane and a negative charge on the inside. When a stimulus reaches the surface, a breakdown of resistance occurs; the permeability for the negative ion is increased, resulting in a depolarization. The depolarized point of the membrane is negative to the adjacent region; whereby a small electric current, the "Strömchen" of HERMANN, is generated. This current in its turn stimulates the adjacent region, leading there to a depolarization. The same process is repeated in successive parts of the nerve fibre and in this way the impulse is propagated along the axon.

Recent developments have made necessary a modification of the membrane theory in its original form. It has been shown by Curtis and Cole<sup>7</sup> and by Hodgkin and Huxley<sup>8</sup> that during the passage of the impulse there occurs not only a depolarization but an actual reverse of the charge. This result was obtained in experiments on the giant axon of Squid by the introduction of an electrode into the interior of the axon and by direct determination of the potential across the membrane. The spike potential was found to be markedly greater than the potential difference in rest, in some cases it was nearly twice as great. There are some technical difficulties which make the exactness of the absolute values uncertain, but the fact that the charge is reversed during activity appears to be unquestionable and well established. It follows that the assumption of a simple depolarization cannot be maintained. The process responsible for the generation of the flow of current is complex and is not merely an abolition of the resting potential.

The availability of radioactive ions made possible the study of the movement of ions across the neuronal surface membrane. Such investigations were initiated during the last two years by Hodgkin and Hunley and Keynes in England and by Rothenberg in this laboratory. The results will be fully discussed in the following paper. They show that sodium and potassium ions are being constantly exchanged, the latter at least to some degree between the inside of the axon and its outer environment. The ionic equilibrium is a dynamic and not a static condition. The conclusion is similar to that encountered in many other fields where radioactive or stable isotopes were used (Schoenheimer<sup>12</sup>).

During activity the outflow of potassium and the influx of sodium are greatly increased. The data of the two laboratories are in good agreement and supplement each other. According to the Cambridge group about  $2 \cdot 10^{-12}$  mole of potassium leaks per cm² surface per impulse; Rothenberg's experiments indicate that the influx of sodium is about  $4 \cdot 10^{-12}$  mole per cm² per impulse. The question how this movement of the two species of ions in opposite direction may account for the reverse of the charge is still open. No satisfactory hypothesis has been advanced so far. It is obvious, however, that events must take place in the active membrane, the site of the electrical manifestations,

which make this accelerated ionic flow possible, and others which restore the resting condition. Experimental evidence that such events actually take place during the passage of the impulse has been obtained by observations of Cole and Curtis<sup>13</sup> carried out with the giant axon of Squid. These investigators measured the impedance changes with alternating current of varying frequency applied across the nerve fibre. The impedance was always reduced during the passage of the impulse. Analysing their results, they concluded that the membrane resistance breaks down during activity from about 1000 ohms per square centimeter to about 40 ohms per square centimeter.

The assumption of a process in the membrane responsible for the electrical manifestations is not in contrast but in full agreement with all classical views. As was stated by Keith Lucas and Adrian<sup>14</sup> more than 30 years ago, all facts indicate that the energy for the propagation of the nerve impulse cannot be derived from the stimulus itself as in the case of a sound wave. According to the English investigators the energy must be supplied locally by a "propagated disturbance". The most likely assumption as to the nature of the "propagated disturbance" is that of a series of chemical reactions producing a change of the proteins or lipoproteins of the membrane and resulting in an increased permeability. Some kind of trigger mechanism must be responsible for the change by which the ionic concentration gradient, inactive in rest, becomes effective. This concentration gradient appears to be the most probable source of the electromotive force. The change in the membrane required for this process must be, from the thermodynamic point of view, associated with an irreversible loss of energy. The reversal will require energy supply which can be conceivably derived from chemical reactions only. It is remarkable that Keith Lucas (l.c.) in logical conclusion of his views postulated that conduction must be associated with heat production, although at that time all attempts to demonstrate it had failed. In 1926, however, A. V. HILL and his associates were able to demonstrate heat production associated with nerve activity after they had developed the recording instruments to an amazingly high degree of perfection<sup>15</sup>. In the same year evidence was obtained by Gerard and Meyerhof that conduction is accompanied by extra oxygen uptake<sup>16</sup>.

These investigations have established the experimental basis for the assumption that conduction is associated with chemical reactions. The finer mechanism, however, remained unknown. A. V. HILL'S LIVERSIDGE lecture: Chemical Wave Transmission in Nerve, delivered in 1932, was a challenge to biochemists to approach this central problem of neurophysiology<sup>15a</sup>. Without a satisfactory answer as to the nature of the chemical changes generating the flow of current, no decisive progress in the understanding of the mechanism of nerve function will be achieved. The difficulty of finding this answer is easily understood if we consider the information obtained by the physical recordings. The initial heat per gram nerve per impulse in a frog sciatic nerve is of the order of magnitude of 10<sup>-8</sup> gcal. The chemical reactions involved in the primary event must take place within one-tenth of a millisecond or less. Reactants in a process of such a high speed, metabolized in amounts of such a small order of magnitude, cannot be measured directly.

OTTO MEYERHOF'S pioneer work on muscular contraction has shown how much information as to the mechanism of cellular function may be obtained by the study of enzymic reactions and by correlating them with events recorded with physical methods on the living cell. By the successful linking of cellular metabolism and function MEYERHOF's work opened new pathways and was perhaps still more revolutionary than in other fields.

It was under the inspiration obtained in Professor Meyerhof's laboratory that References p. 93/95.

the writer has tried to approach the problem of nerve metabolism in relation to function in a way similar in principle to that which had proved so satisfactory and valuable in the study of muscular contraction. It is a particular pleasure and privilege to pay tribute to Professor Meyerhof at the occasion to which this volume is dedicated by reviewing some aspects of this work.

# Role of Acetylcholine in Conduction

Since the discovery of the powerful pharmacological effects of acetylcholine by Reid Hunt and Taveau<sup>17</sup> early in this century, the compound has attracted the attention of physiologists. Observations of Magnus, Dale, Loewi, Cannon and many others suggested that acetylcholine may be released from nerve endings and act as a "mediator" of nerve impulse to the effector organ. There were many difficulties and contradictions and the theory of chemical mediation encountered increasing opposition (Fulton<sup>18</sup>, Eccles<sup>19</sup>).

During the last 14 years the writer and his associates have offered evidence indicating that the theory in its original form has to be modified. Based on the approach outlined above, a great variety of facts have accumulated suggesting that the release and removal of acetylcholine are intracellular processes<sup>20–23</sup>. They seem to be closely associated with the alterations in the active membrane which occur during the passage of the impulse. The transmitting agent is the flow of current but in the chain of events which generate the "Strömchen" the acetylcholine-esterase system appears to play an essential role.

The important data have recently been summarized at a Symposium on the physiological role of acetylcholine<sup>23</sup>. A more detailed and comprehensive presentation may be found in the textbook on Hormones<sup>24</sup>. It may suffice to mention here briefly a few essential facts, supporting the assumption of the necessity of acetylcholine in conduction.

Studies on the enzyme which hydrolyses acetylcholine, acetylcholine-esterase, have revealed the following features: I. The reaction occurs at an extremely high rate, the "turnover number" is 20 000 000 per minute or even higher, indicating that one molecule of ester may be hydrolysed in 3-4 millionth of a second<sup>25</sup> or possibly even faster (unpublished data). This high speed is pertinent for any assumption correlating a chemical reaction directly with the electrical manifestations of conduction. 2. Acetylcholineesterase is present in all conducting tissues throughout the whole animal kingdom<sup>26, 27</sup>. 3. The enzyme is localized exclusively in the surface where the bioelectrical phenomena occur. This is in contrast to many other enzymes required for conduction, as for instance the respiratory enzymes<sup>28</sup>. 4. The concentrations of the enzyme are adequate to account for an amount of acetylcholine metabolized which is compatible with the assumption of an essential role in conduction. 5. The enzyme in conducting tissues has a number of properties by which it may be easily distinguished from other esterases occurring in the organism<sup>26, 29</sup>. Only in erythrocytes the same type of esterase is found. Since the physiological substrate is known to be acetylcholine, the use of the term acetylcholineesterase for this enzyme has been recently proposed<sup>30</sup>.

All these features of acetylcholine-esterase, however suggestive, would not yet permit the assumption of its essentiality for conduction. The enzyme activity has, however, been correlated in many ways with the electrical events of conduction. In experiments on the electric organ of *Electrophorus electricus* a direct proportionality has been established between the voltage of the action potential and the concentration of

acetylcholine-esterase over a wide range, varying from 0.5 to 22 volts per cm<sup>31</sup>. No other enzyme tested shows any parallelism. The result supports the assumption of a close relation and interdependence between these electrical and chemical processes.

Using the same material, it has been shown that the energy released by the breakdown of phosphocreatine is adequate to account for the total electrical energy released by the action potential. It appears probable that phosphocreatine acts, as in muscle, only as a reserve for energy rich phosphate and that the breakdown of adenosine triphosphate (ATP) precedes that of phosphocreatine. In contrast to muscular contraction, however, it appears for many reasons unlikely that ATP may be the primary reaction associated with conduction <sup>23, 24</sup>. If the postulate that acetylcholine may be directly associated with conduction is correct, the hydrolysis of the ester should precede the breakdown of ATP and the energy released by the latter used for the synthesis of acetylcholine. In accordance with this postulate, an enzyme, choline acetylase, was extracted from brain which in cell free solution synthesizes acetylcholine using the energy of ATP<sup>32</sup>, <sup>33</sup>. It was the first demonstration that acetylation, occurring so frequently in intermediate metabolism, requires ATP energy and, more generally, that ATP energy may be used outside the glycolytic cycle, in which its crucial role had been shown, first by MEYERHOF and his associates and later extended by the work of PARNAS, the CORIS, NEEDHAM, SZENT-GYÖRGYI and many others.

Finally it has been shown with a great variety of conducting tissues, nerve and muscle, that inactivation of acetylcholine-esterase by specific inhibitors results in an abolition of conduction<sup>27, 34</sup>. This effect is easily reversible with compounds which inhibit the enzyme reversibly. With DFP, an inhibitor which inactivates the enzyme irreversibly, the abolition of conduction becomes irreversible. However, the irreversible inactivation of the enzyme is a relatively slow process. Its rate depends on a great number of factors<sup>35</sup>. Therefore, this compound was particularly suitable for testing the essentiality of acetylcholine in conduction. A striking parallelism has been established in nerves exposed to DFP between the progressive inactivation of acetylcholine-esterase and the abolition of conduction as a function of time and temperature. In no way is it possible to dissociate conduction from acetylcholine-esterase activity<sup>36, 37</sup>. Claims to the contrary were shown to be due to the use of inadequate techniques. The minimum amount of enzyme required for unimpaired conduction is relatively small, about 10% of the total activity present. Considering the smallness of the initial heat, the remaining activity is, however, still adequate<sup>38</sup>. The excess is not unusual and is in accordance with the experience with other enzymes, but it led to some misinterpretations in the early phase of the investigations.

The view that the acetylcholine-esterase system is essential in conduction appears to be well established. The precise function of the ester is, however, unknown. It is possible that, during activity, a higher rate of collision of sodium or potassium ions with the acetylcholine-protein or lipoprotein complex leads to a release of the ester. This process may be an essential factor in the alterations of the membrane proteins leading to an increased permeability. The possibility of a rapid removal of the active ester by acetylcholine-esterase which would restore the resting condition permits such an assumption. No other process is known to have the necessary speed. An electrogenic action of the ester may be demonstrated in electric tissue, as will be discussed later. In connection with the great number of other electrical and chemical observations the hypothesis appears worthy of consideration. In this connection, the experiments reported in the following paper on

the effect of inhibitors of acetylcholine-esterase on the ion permeability are also of interest although still far from conclusive.

It was mentioned above that the esterase in the red blood cell has the same characteristic features as the esterase in conductive tissue. There, too, the enzyme is localized exclusively in the surface membrane<sup>39</sup>. It is therefore of interest that Greig and Holland<sup>40</sup> have described observations suggesting that inhibitors of choline ester splitting enzymes may affect the permeability of red blood cells. If this hypothesis be confirmed, it will be another support for the assumption of a similar function of acetylcholine in the neuronal surface membrane. Analogies as to the permeability of these two types of cells have long been known to physiologists.

# Difference between conduction and synaptic transmission

In view of the evidence that acetylcholine has an essential function in conduction it appears necessary to reconsider the role of the ester in synaptic transmission. It is the purpose of this article to analyse the question how the earlier observations, suggesting the theory of chemical mediation, may be integrated into the picture resulting (I) from the enzyme studies and (II) from the attempt to correlate the chemical and physical events of nerve activity.

The theory of chemical mediation was based essentially on two facts: 1. the stimulating effect of acetylcholine in relatively small amounts (a few  $\mu g$ ) upon synaptic junctions, and 2. the appearance of acetylcholine in the perfusion fluid of such foci following nerve stimulation. The complete inertness of the fibre to acetylcholine even if applied in high concentrations (up to 20 g per liter) was considered as definite proof that the physiological function of the ester is limited to the synapse.

a) Impermeability of the axonal surface membranes to acetylcholine. Studies on the permeability of the axonal surface membranes have thrown new light on this problem and have provided a satisfactory explanation for the discrepancy between the earlier observations and the conclusions necessitated by the enzymatic studies. The investigations were carried out on the giant axon of Squid. This material is unusually favourable in view of the large diameter (0.5 to 0.7 mm) of the axon. It is possible to extrude the axoplasm from the cell interior of this preparation without contamination by substances attached to the outside surface. The axoplasm thus obtained may be analysed for compounds to which the axon has been exposed for various periods of time. In this way the inside concentration of these compounds and if desired the rate of penetration may be determined.

It was found that those inhibitors of acetylcholine-esterase which alter and abolish conduction, like eserine and DFP, penetrate into the axoplasm, although the rates of penetration of the different compounds may vary considerably<sup>37</sup>. In striking contrast to the compounds mentioned prostigmine, an extremely potent inhibitor of acetylcholine-esterase, does not affect conduction even in high concentrations (10<sup>-2</sup> M)<sup>34</sup>. This compound was not found in the axoplasm, although the methods used were highly sensitive and adequate to detect an extremely small fraction of the concentration of the compound present on the outside. The experiments show that the axonal surface membranes are impervious to prostigmine and, moreover, that the site of the acetylcholine-esterase associated with conduction must be inside a structural barrier which makes the enzyme inaccessible to the inhibitor. Eserine is a tertiary amine and lipid soluble, prostigmine is a quaternary ammonium salt and lipid insoluble. It appears likely that the difference

in chemical structure and properties is responsible for the difference in permeability of these two types of compounds. Possibly the lipid membrane, known to surround all axons, whether myelinated or not, may be the structural barrier.

Acetylcholine like prostigmine is a methylated quaternary ammonium salt. The failure of acetylcholine to affect conduction was explained by the assumption that the axonal surface membrane may be impervious to the choline ester. This assumption has been tested directly in the following way. The axons were exposed to acetylcholine labelled with N<sup>15</sup>. High concentrations (20 gram per liter) were used. When the axoplasm was tested for the presence of N<sup>15</sup>, only insignificant traces were present. These traces, moreover, were largely accounted for by the contamination of the acetylcholine used with tertiary amine containing N<sup>15</sup>. Tertiary amine labelled similarly with isotopic N penetrated rapidly and an equilibrium between the inside and outside concentration was obtained within 60 minutes<sup>41</sup>. Fig. 1 demonstrates the results obtained.

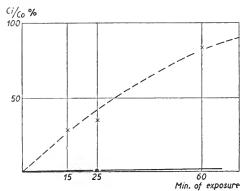


Fig. 1. Rate of penetration of trimethylamine and acetylcholine labelled with  $N^{15}$  into the interior of the giant axon of Squid. The ratio of the concentration of the N of these compounds inside (Ci) to that outside (Co) is plotted against the time of exposure in minutes. The dotted line indicates the rate of penetration of N on exposure to trimethylamine (280  $\mu g$  N per ml), the straight line, that of the N found on exposure to acetylcholine (1430  $\mu g$  N per ml of which 55  $\mu g$  were non-quaternary N)<sup>41</sup>.

The experiments show conclusively that the axonal surface membranes are impervious to acetylcholine. They explain why the fibre remains inert when the ester is applied externally, even in high concentrations. The fact that the action of the ester is limited to the synaptic junction indicates that the active membrane may be reached at these foci even by those compounds which do not penetrate into the interior of the axon or the muscle fibre. The peculiar ability of the synapse to react to compounds which do not affect axonal conduction appears thus to be due to a difference in anatomical structure. This applies also to curare which, as recent observations have shown (King<sup>42</sup>, Wintersteiner and Dutcher<sup>43</sup>), has as active principle a methylated quaternary ammonium salt. The observation of CLAUDE BERNARD that this compound acts exclusively on the neuromuscular junction and does not affect nerve or muscle fibres

was for a century the basis underlying the assumption that the neuromuscular junction has special properties. It seemed to support the view that the fundamental mechanism of transmission may differ from that of conduction.

On the basis of the investigations described, the schematic presentation of the neuromuscular junction in Fig. 2 may serve as illustration of the situation. Only the compounds on the left side are capable of acting everywhere, because they may penetrate through the structural barriers. In contrast, the compounds on the right side act only upon the post-synaptic membrane which appears to be either less or not at all protected. The nerve ending itself, although not surrounded by myelin, appears also to be protected by a structural barrier since, according to  $\text{Bronk}^{44}$ , it is inexcitable even by relatively high concentrations of acetylcholine in the perfusion fluid.

Recently it was found that tetraethyl pyrophosphate (TEPP) does not affect con-

duction<sup>45</sup>. TEPP is an extremely potent inhibitor of acetylcholine-esterase, much more powerful than eserine, prostigmine and DFP. TEPP inactivates the enzyme irreversibly like DFP but this effect is immediate, in contrast to the slowly progressive action of

DFP46. Nevertheless, in a frog sciatic nerve exposed to TEPP in concentrations (2 mg per ml) several thousand times as high as those required to inactivate completely and irreversibly the enzyme in solution, conduction remains intact. This suggests that the acetylcholineesterase retains its activity. Under the same conditions DFP which penetrates into the interior abolishes conduction and enzyme irreversibly, although it is thousand times less potent as inhibitor. The only apparent explanation for the failure of TEPP to penetrate into the axon is its insolubility in lipid. Since this property applies also to methylated quaternary ammonium salts, the assumption gains further support that the structural barrier may be a lipid membrane surrounding nerve and

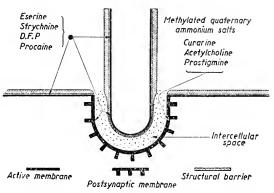


Fig. 2. Scheme of the neuromuscular junction. A structural barrier protects nerve and muscle fibre against the action of methylated quaternary ammonium salts. These compounds act only on the postsynaptic membrane, which apparently is either less or not at all protected. Other compounds, like eserine, DFP, strychnine, and procaine, being able to penetrate through the structural barrier, act upon the active membrane of nerve and muscle fibre<sup>23</sup>.

muscle fibre but absent at the post-synaptic membrane of synaptic junctions. But whatever the anatomical location and the chemical nature of the barrier may finally turn out to be, it is of decisive importance to recognize its existence. The barrier has not been identified morphologically but has to be postulated on the basis of the physico-chemical and enzyme studies described.

It has been reported that intact nerves may split at least 25% or more of the acetylcholine which may be hydrolyzed during the same period by the ground nerve<sup>47</sup>. On the basis of this result, it was concluded that acetylcholine may penetrate into the interior. Since it has been shown that acetylcholine does not penetrate into the axon, even if applied in high concentrations, the more likely conclusion from this observation is the location of part of the enzyme outside the barrier. It has never been claimed that all the esterase present is inside and necessary for conduction. The experiments reported<sup>47</sup> were carried out with the manometric technique in which the CO<sub>2</sub> output is measured. There has recently been introduced by HESTRIN a new simple and rapid chemical method which makes possible a direct determination of the acetylcholine removed by hydrolysis<sup>48</sup>. This method is based upon the reaction of O-acyl groups with hydroxylamine in alkaline medium. It is more specific than the manometric method, especially when large amounts of tissue are necessary and simultaneous chemical reactions cannot be excluded. Using this method it has been found that the acetylcholine-esterase activity of the ground nerve is about twice as high as the manometric method indicates. The intact nerve splits acetylcholine at a rate which is only a small fraction (about 5 to 7%) of the total activity<sup>45</sup>. This activity is suppressed by prostigmine which like acetylcholine does not penetrate into the interior. Complete inhibition of this enzyme activity does not affect conduction. The meaning of the small amount of esterase on the outside

of the barrier is not clear. The activity may be due to an unspecified esterase other than acetylcholine-esterase or to the presence of small blood vessels, microscopic muscle fibrils or cut nerve fibres where the surface may be reached by the ester. This is, however, entirely irrelevant for the major problem involved.

The elucidation of the situation became possible by the fortunate circumstance that so many different kinds of extremely potent inhibitors of acetylcholine-esterase were available: reversible and irreversible types of inhibitors and in each of the two groups compounds which penetrate and others which do not penetrate. This combination made it possible to find a satisfactory answer to some of the most pertinent questions involved: I. the necessity of acetylcholine-esterase for conduction; 2. the existence of a barrier for methylated quaternary ammonium salts, and 3. the localization of the enzyme in respect to the barrier.

Even if a compound affects both axon and synapse, there may still be a great difference as to the concentration required. Chemical substances may act upon the apparently unprotected active surface of the post-synaptic membrane in concentrations much smaller than those necessary for affecting the nerve or muscle fibre. An interesting illustration is provided by the experiments of ROEDER and his associates49, who found that DFP abolishes synaptic transmission in much lower concentrations than those which affect conduction. DFP is very lipid soluble and may therefore accumulate in the myeline sheath to a certain concentration before penetrating into the aqueous interior of the fibre in concentrations sufficiently high to inactivate the enzyme and, consequently, to abolish conduction. At the time when conduction disappears, the concentration of DFP is small in the axoplasm compared with that in the outside fluid<sup>37</sup>. This finding supports the assumption that the concentration of DFP at the site of action may be small and is consistent with the potency of the compound as inhibitor of acetylcholine. The necessity of a high outside concentration may be attributed to the relatively slow rate of penetration. In the case of eserine, the distribution between inside and outside at the same period, i.e., at the time when the action potential has disappeared, is very different. The rate of penetration will be determined by the properties of the various chemical compounds on the one hand and by the properties of the various surface membranes. Additional factors may be of importance, such as the affinity of the compound to the enzyme, its potency as inhibitor and the kinetics of the inhibition. In view of the complexity of the process, it is not surprising that in applying potent inhibitors of acetylcholine-esterase, the phenomena observed may differ sharply in so many respects, although the underlying cause is the same chemical reaction.

The action of procaine, one of the compounds marked on Fig. 2, requires comment. The blocking of conduction by this and other similar anaesthetics cannot be explained in terms of acetylcholine-esterase inhibition. These compounds are weak inhibitors of acetylcholine-esterase, although other esterases may be affected more strongly<sup>50</sup>. Thimann<sup>51</sup> has pointed out that these compounds have some resemblance in structure to acetylcholine, but are tertiary amines. They will, therefore, easily penetrate into the interior and they may act competitively with the ester on some proteins or lipoproteins of the membrane. Since apparently they do not depolarize the membrane<sup>52</sup>, it is possible to assume that they form a complex but, in contrast to acetylcholine, they do not change the condition of the protein. However, they may prevent the action of the ester released and thereby block conduction, whereas otherwise the resting condition may remain unchanged. This is consistent with the apparent failure of cocaine,

described in the following paper, to produce a significant change in permeability.

b) Release of acetylcholine during activity. In view of the permeability studies described, the limitation of the action of acetylcholine to the synapse, if the ester is applied externally, cannot be used as an indication for a special role at this junction, as was proposed by the theory of chemical transmission. For the same reason, the second fact on which the hypothesis was built has to be reconsidered. The appearance of acetylcholine in the perfusion fluid of the synapse following nerve stimulation must be attributed to the absence of an insulating membrane. If acetylcholine cannot pass through the structural barrier into the interior, it will not be able to leak from the inside to the outside in stimulated nerve and muscle fibres. The only site where such leakage will be possible is the postsynaptic membrane. However, even at the synaptic junction the ester does not appear under physiological conditions. DALE and his associates have repeatedly emphasized that the ester appears in their experiments only if the normal mechanism responsible for the rapid removal of the ester, viz., acetylcholine-esterase, is largely inactivated by the presence of eserine. Even in presence of the drug, the amounts leaking out were extremely small, about one hundred-thousandth of that required to set up a stimulus. On the basis of more recent experiments, in which acetylcholine was applied directly to the motor end plate, the difference was of the same order of magnitude. Such a difference is not easily explained in terms of chemical mediation. It is true that in Loewi's original observations on the frog heart, no eserine was present. However, considerable difficulties were encountered by him as well as other investigators when they tried to reproduce the appearance of the ester. For this reason, Loewi's theory was repeatedly criticized<sup>53, 54</sup>. When a heart preparation has been perfused for a certain period of time with Ringer's solution, the post-synaptic membrane may not be in a completely normal condition and may therefore permit leakage of the compound, which under physiological conditions may be rapidly inactivated. The condition of the membrane may depend upon a variety of factors, such as the length of the perfusion period, the composition of the perfusion fluid, the condition and the species of the frog used, etc. Variations of these factors may explain the difficulties encountered by a number of investigators who tried to reproduce this observation. The same consideration may be applied to the finding of Kibjakow<sup>55</sup>, who in 1932 described the appearance of acetylcholine in the perfusion fluid of the synaptic ganglion in absence of eserine. His observations were questioned by Dale's school, but it is conceivable that with the less perfect perfusion technique in Kibjakow's experiments, the active membrane suffered more damage and thus permitted the leakage of traces just in the measurable range. So far there is no conclusive evidence that the appearance of the ester outside the cell is a physiological event.

It is an interesting psychological phenomenon, encountered frequently in the progress of science as well as in the work of individual investigators, that certain observations are neglected or even discarded because they are inconvenient, puzzling and do not fit into preconceived ideas. Later, when the views have changed, the facts may suddenly gain significance and it becomes possible to integrate them into the general picture. The release of acetylcholine at the synapse assumes a new aspect if considered in connection with other pertinent observations which at the time of their presentation did not find sufficient attention.

In 1933, simultaneously with or even prior to the finding of Dale that acetylcholine appears in the perfusion fluid of the sympathetic ganglion or of the neuromuscular juncture.

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tion, CALABRO<sup>56</sup> had shown that, following prolonged stimulation of the rabbit vagus, an acetylcholine-like substance is released from the cut end into the surrounding fluid. BINET AND MINZ<sup>57</sup> found, in 1934, that from the transsected surface of nerves a compound is released which increases the sensitivity of the leech muscle to acetylcholine. Calabro's findings were confirmed and extended by Bergami<sup>58</sup> and by Babski and Kisljuk<sup>59, 60</sup>. In 1937 VON MURALT<sup>61</sup> described a difference of the acetylcholine content between stimulated and unstimulated nerves. In view of the possibility of a very rapid disappearance of the active ester, he developed a special technique by which he "shot" the nerves into liquid air. Tested by bioassay after a short period of extraction the amount of acetylcholine was 0.2 µg per gram in the stimulated as compared with 0.12 µg per gram in the control nerve. In a large series of experiments the difference between stimulated and control nerve was later found to be 0.00  $\mu g$  per gram<sup>62</sup>. However, the difference between the two nerves disappears if extraction is continued for a longer period of time. There is, therefore, some uncertainty as to the interpretation. It is conceivable that the acetylcholine released from its complex is present in a free form and therefore diffuses from the frozen tissue during extraction more rapidly than that part of the acetylcholine which is bound to protein or lipoprotein.

Even in sensory nerves release of acetylcholine has been demonstrated by BRECHT AND CORSTEN<sup>63</sup> from the cut end after stimulation. These investigators used a remarkably sensitive method, the contraction of the frog lung in presence of eserine, and hereby succeeded in detecting the ester released. The amounts are still smaller than those released from motor nerves, but this difference appears consistent with the smaller rate of metabolism indicated by the lower concentrations of acetylcholine-esterase and choline acetylase<sup>27</sup>. It is significant that the release of acetylcholine has been demonstrated in parasympathetic, motor and sensory nerve fibres. The situation is pertinent in connection with the finding that the enzymes which form and hydrolyse acetylcholine are present in all types of nerves and that the inactivation of acetylcholine-esterase invariably leads to abolition of conduction.

The facts described support the assumption that there is no difference in principle between the release of acetylcholine at the synapse and in the axon, except that in the latter case the ester cannot pass through the structural barrier. They make it appear still more probable that this release is an intercellular process and that the appearance outside the cell at the synapse must be attributed either to the poisoning of the enzymic mechanism, normally preventing the leakage or to some other damage of the active surface where it is least protected and most vulnerable. At the time when these findings were described, acetylcholine was considered to be a chemical mediator and since chemical transmission in the axon is inconceivable, it was difficult to integrate them into the general picture. Little or no attention was consequently paid to these findings. VON MURALT has been very cautious in his statements as to the possible significance of the release of acetylcholine in the nerve fibre. He called the ester an "Aktionssubstanz", meaning that it may be important like many other substances for nerve activity in the axon as well as at the synapse. This caution was well justified at a time when nothing was known about the high speed of the reaction, the effects of acetylcholine-esterase inhibitors on conduction and the great variety of other factors known today. These facts had to be established before it became possible to assume a direct association of the ester with the generation of the electric currents which propagate the impulse. In the light of recent developments, however, the situation

has changed. The demonstration of the release of acetylcholine in the axon appears as relevant as that at the synaptic junction and requires a modification of the original interpretation.

The structural barrier for acetylcholine present in the fibre and its absence in the post-synaptic membrane may be considered as the main reason that the attention of many physiologists was focused for such a long time on the synapse only. Very little is known concerning the properties of the barrier and the factors affecting it. The observations on the permeability of neuronal surface membranes described in this and the following paper are only an initial phase in the attempt of analysing the problem. Its importance can hardly be overemphasized, not only for the understanding of the cellular mechanism but of the pharmacology and pathology of the nervous system as well. The development of new drugs may be greatly facilitated if the structural factors determining the permeability and the rate of penetration are known. In many cases an action may be desirable, preferably or exclusively, on the synapse, in others, upon both axon and synapse.

The existence of structural barriers and the great variations of their properties may account for the many obstacles encountered and the many contradictory reports when the two criteria of chemical mediation were applied to different types of synapses. The unnumerable differences of anatomical structure, the biochemical composition of the surrounding medium and many other accessory conditions must be essential in determining the action of acetylcholine when applied externally. These variations do not permit the assumption that the fundamental physico-chemical mechanism of the propagation of the nerve impulse may not be the same. In view of the physico-chemical properties of acetylcholine and similar N-methylated compounds, the difficulties will become nearly insurmountable in the study of brain and spinal chord which contain large amounts of lipid. It is not surprising that the painstaking efforts to demonstrate or to disprove the "cholinergic" nature of synapses in brain and spinal chord have resulted in a most unsatisfactory and confusing picture.

In contrast the conflicting results obtained when the "cholinergic" nature of synapses, especially in brain, is tested by the usual criteria of chemical mediation, the approach based on the study of the enzymes connected with acetylcholine metabolism and their correlation with function did not encounter comparable difficulties. All results obtained in this way indicate the generality of the role of acetylcholine in all conducting tissues, including that of brain and spinal chord<sup>24</sup>.

c) Basic similarity between conduction and transmission. At the Symposium on the synapse, in 1939, Erlanger<sup>64</sup> scrutinized the problem whether the electrical characteristics of synaptic transmission are basically different from those which may be observed on the axon. His data indicate that the electrical phenomena considered to be pecularities of the synapse may be demonstrated on fibres, viz., latency, one-way transmission, repetition, temporal summation and facilitation, and transmission of the action potential across a non-conducting gap. The facts based on the electrical signs of nerve activity make it unnecessary to assume that any condition exists at the synapse which differs in principle from that found in the peripheral axon, except in quantitative respect.

Ten years have passed. During that time extensive investigations have been made on the electrical characteristics of transmission across the natural and artificial synapse (ephapse). From the work of many investigators, mainly ARVANITAKI<sup>65, 66</sup>, BULLOCK<sup>67</sup>, ECCLES<sup>19</sup>, GRANIT AND SKOGLUND<sup>68</sup> and others considerable evidence has accumulated

in support of Erlanger's views that the basic mechanism of transmission and conduction is the same, the propagating agent being in both cases the flow of current. According to Eccles<sup>19</sup>, impulses travelling down the pre-synaptic fibre, generate a current which produces in the synaptic membrane of the post-synaptic cell an anodal focus with cathodal surround; this is followed in a second phase by a more intense cathodal focus with anodal surround. The cathodal focus sets up a local response from which a catelectrotonus spreads over the post-synaptic cell membrane. The catelectrotonus, the end plate potential, sets up a propagated impulse in the post-synaptic cell as soon as a certain threshold is reached. The sequence of events is similar to that observed on artificial synapses and on a single unit preparation of the synapse, the stellate ganglion of Squid (Bullock<sup>67</sup>). Since the electrical signs and the biochemical data favor the assumption that the mechanism of transsynaptic transmission is basically the same as that of conduction, it follows that the role of acetylcholine in these mechanisms is most likely the same. In both cases the propagating agent is the flow of current, but the release and the removal of acetylcholine must be essential events in the alteration of the pre- and post-synaptic membrane during the flow of current across the synaptic region and the generation of the end plate potential. It would be difficult to picture these currents as being different in nature from those in the axons. A few biochemical data may be mentioned in this connection which support the assumption of a high rate of acetylcholine metabolism in the post-synaptic membrane of the motor end plate. COUTEAUX AND NACHMANSOHN<sup>69, 69a</sup> found that, following the section of the sciatic nerve of guinea pigs, the high concentration of acetylcholine-esterase of the motor end plates of the gastroncemius decreases only slightly. Within three to four weeks after the operation one-fourth or possibly less of the enzyme concentration had disappeared. Then the activity remains constant for many months. This result suggests that three quarters of the enzyme or more is localized in the post-synaptic membrane, the "sole plate" of KÜHNE, a pure muscular element which persists after the disappearance of all nerve elements.

The electric organs have physiologically evolved from striated muscle. The electric plates are homologous with the motor end plate. The discharge of these organs is homologous with the end plate potential. Recent studies of Couteaux<sup>69b</sup> have revealed that the post-synaptic membrane of the motor end plate is morphologically a very peculiar structure. By using Janus green or methyl violet, he demonstrated a striking similarity with the electrolemma of the electric plate surrounding the nerve endings. The direct proportionality between the voltage developed during the discharge and the concentration of acetylcholine-esterase observed in the electric tissue suggests a high rate of acetylcholine metabolism associated with the end plate potential.

These findings alone without all the other evidence accumulated would not necessarily imply that the acetylcholine is released in the post-synaptic membrane itself. The following observations are, however, of interest in this connection. The discovery of the extraordinarily high concentration of acetylcholine-esterase in electric tissue made possible the assumption that acetylcholine might be the agent that produces the depolarization presumably occurring during the action potential. The possibility of a depolarizing action of acetylcholine has been considered by Dubuisson and Monnier<sup>70</sup> and Cowan<sup>71</sup>. In 1938, when the prerequisite for such a theory, namely the high speed of destruction of the active agent appeared established, Auger and Fessard tested the effect of eserine on the discharge of the electric tissue of *Torpedo marmorata*<sup>72</sup>. As may be seen in Fig. 3

the height of the potential is markedly depressed in presence of eserine. The duration of the descending phase is considerably prolonged. This effect of eserine on the end plate

potential is consistent with the assumption that the appearance and the removal of acetylcholine within the post-synaptic membrane may be essential for the generation of the potential.

In view of their corresponding biochemical and bioelectrical findings, Fessard and Nachmansohn decided then to test whether acetylcholine injected into the electric organ may produce an action potential. Such an electrogenic effect might be expected if acetylcholine is the compound which is responsible for the alterations of the membrane, occurring during the discharge. In experiments carried out at Arcachon in 1939 on *Torpedo marmorata*, in which they were joined by Feldberg,

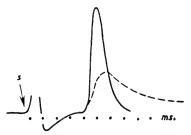


Fig. 3. Effect of eserine on the discharge of electric tissue of *Torpedo marmorata*. The fully drawn line shows the discharge in absence, the dotted line in presence of eserine<sup>7</sup>.

they were able to demonstrate that acetylcholine has an electrogenic effect<sup>73, 74</sup>. The arterial injection of acetylcholine caused potential changes similar to the natural discharge. However, the changes were small and slow and very large amounts were necessary for the effect. Fig. 4 illustrates the effects of acetylcholine injected in amounts varying between 5 and 200  $\mu$ g. 5  $\mu$ g had no effect. With 200  $\mu$ g the potential difference was about 0.7 millivolts and the descending phase had not yet reached the base line after several seconds. If the acetylcholine is injected in presence of eserine, preventing a too rapid destruction of the ester, the effects are greatly enhanced. Fig. 5 shows that under these conditions an effect may be obtained even with 2.5  $\mu$ g of acetylcholine. With 10  $\mu$ g the potential change produced is greater than 3 millivolts, although the duration is still about 3 seconds.

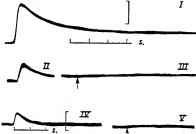


Fig. 4. Potential changes produced by intraarterial injection of acetylcholine into the electric organ of *Torpedo marmorata*. I, II, IV and V correspond to the injection of 200, 100, 20 and 5  $\mu$ g of the ester; whereas at III only perfusion fluid was injected. Between II and III the sensitivity has been increased fourfold. 0.5 millivolt indicated at I, 0.1 millivolt at IV. Time in seconds.

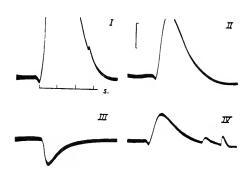


Fig. 5. Potential changes produced in the same way as in Fig. 4 but in presence of eserine. I, II and IV correspond to the injection of 10, 5 and 2.5  $\mu g$  of acetylcholine; at III only perfusion fluid was injected. 0.5 millivolt indicated at II. Time in seconds.

The experiments show that the ester may produce an alteration of the membrane preceding the flow of current. They support the view that the ester plays an essential role in the generation of the current and make it difficult to assume that the release of References p. 93/95.

acetylcholine may occur in the recovery period. In that case it would be hard to understand how the compound produces current. Although the potential changes resemble the normal discharge, there is, however, a most striking contrast in two respects: the smallness of the voltage and the 1000 fold increase of the duration. The normal discharge occurs in 2 to 3 milliseconds; the voltage of a single unit is about 100 millivolts. Although a quantitative evaluation is impossible since the number of units in series reached by the intraarterial injection is uncertain, the discrepancy as to duration and strength is enormous, even in presence of eserine. The method used is crude compared to the effect which might be expected if the compound were released from the nerve ending. In that case it would reach the opposite surface much faster, but in view of the relatively large amounts injected, of which apparently at least a fraction reaches the active membrane, the response is small beyond all proportion. It thus becomes difficult to conceive that physiologically the substance is released from the nerve ending and, penetrating the intercellular space, produces the end plate potential. This difficulty does not arise if it be assumed that the release and the removal of the ester are intracellular events which do not involve any diffusion but occur in the post-synaptic membrane and generate there the flow of current.

If locally supplied energy is necessary for the small electric currents which propagate the impulse along the axon as postulated by Keith, Lucas, and Adrian, it appears almost certain that such energy will be required for the generation of a potential in the second unit. The flow of current reaching the post-synaptic membrane may result in a release of acetylcholine which may act as a trigger in the chain of events and supply the energy for building up the end plate potential. It is remarkable that exactly this mode of action has been proposed by Lapicque<sup>75</sup> in 1936—''l'état d'excitation suscité dans la sole nucléée peut y déclencher une réaction auxiliaire venant fournir le supplément de puissance requise. Tel serait le rôle de l'acétylcholine; c'est exactement le rôle que joue l'amorce dans la technique des explosifs . . . La production de l'acétylcholine serait, dans cette conception, située, non entre le nerf et le muscle, mais dans le muscle lui-même, auquel appartient sans conteste la sole nucléée. Il s'agirait donc strictement parlant, non d'un intermédiaire dans la transmission de l'excitation entre nerf et muscle, mais d'un premier stade, formant relais dans l'excitation musculaire pour assurer sa généralisation à toute la masse du myone''.

The electrogenic effect of acetylcholine injected into the electric tissue is another illustration of the fact that the post-synaptic membrane is not protected against the ester. It is interesting that the effect of curare on electric tissue was a controversial issue for a long time. Recently, however, Auger and Fessard<sup>72</sup> have shown that the effect of curare is regularly reproducible if the permeability factor is taken into account and the drug is applied in adequate form.

Curare, being a methylated quaternary ammonium salt, may act upon the protein of the active membrane as a competitor of acetylcholine. The effect persists since the compound cannot be hydrolyzed but must be removed by diffusion. If the rapid removal of acetylcholine is inhibited by eserine, the result is strikingly similar to that obtained with partial curarization of the end plate, as the experiments of Auger and Fessard have shown. The depression and prolongation of the potential in Fig. 3 must obviously be attributed to the persistence of acetylcholine and with still higher concentrations of eserine a complete "curarization" will be obtained.

As pointed out by Erlanger, conduction along the axon and transmission across References p. 93/95.

synapses may vary as to quantitative aspects. This is not surprising in view of the discontinuity and other structural differences. Although the time relations are similar, there is a synaptic delay of the order of a millisecond. This may be the result of several factors, as e.g., the decreased diameter of the nerve fibre near the ending which may lead to a decreased rate of conduction. Exact measurements of these various factors are difficult, due to obvious technical reasons. However, the quantitative differences between intracellular and transsynaptic propagation are well in the expected range, and none of them requires the assumption of a fundamentally different mechanism.

In conclusion, no convincing evidence exists supporting the idea that acetylcholine assumes a function at the synapse entirely different from that in the axon, *i.e.* is released from the nerve ending, penetrates the intercellular space and acts on the post-synaptic membrane, thus substituting the flow of current as a "chemical mediator". A fundamental rule of scientific thinking requires that one should not assume two different principles without necessity. Work and Work<sup>76</sup> have recently quoted the excellent formulation of this rule by David Hume in his *Treatise of Human Nature*: "To invent without scruple a new principle to every new phenomenon, instead of adapting it to the old; to overload our hypothesis with a variety of this kind, are certain proofs, that none of these principles is the just one, and that we only desire, by a number of falsehoods, to cover our ignorance of the truth". Neither the so-called "electrical" nor the "chemical" concept of synaptic transmission is satisfactory. The interpretation proposed harmonizes both concepts by integrating the progress achieved concerning the structure, the biochemical data and the electrical signs of activity.

The earlier observations on acetylcholine deserve credit for having drawn the attention of physiologists to this compound in connection with nerve activity. However whereas, the ester was first associated with one type of nerve endings, then with a few others, the study of its role by the combination of chemical and physical methods has shown its essentiality in the conduction of nerve and muscle impulses throughout the animal kingdom. The type of approach applied by Otto Meyerhof to studying muscular contraction has proved valuable in obtaining a better understanding of fundamental principles underlying the mechanism of another cellular function vital for life.

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# STUDIES ON PERMEABILITY IN RELATION TO NERVE FUNCTION

# II. IONIC MOVEMENTS ACROSS AXONAL MEMBRANES\*

by

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#### INTRODUCTION

The ionic concentration gradients which exist between the inside and the outside of nerve fibres and their possible role in nerve function have been discussed in the preceding paper. In spite of the importance of this question very little information is available as to the ionic movements across axonal surface membranes in rest and during activity. The investigations on the giant axon of Squid have demonstrated that this material is most suitable for permeability studies. With the increased availability of radioactive ions from the Oak Ridge pile a more direct approach to the problem became feasible. It was thought that precise and more quantitative data might be obtained by subjecting the giant axon of Squid, *Loligo peallii*, to artificial environments in which all or part of a given ionic constituent was replaced in isomolar concentration with its radioactive isotope.

#### **METHODS**

Chemical. Na<sup>24</sup> and K<sup>42</sup>, available from the Oak Ridge pile in the form of the carbonates, were dissolved in the smallest possible volume of distilled water and then converted to the chlorides by the addition of equivalent quantities of dilute HCl. Aliquots of the neutral solution were then transferred to tared vials and evaporated to dryness under infra-red heating lamps. The quantity of salt per vial was determined by weighing and artificial sea water was prepared from these as described below. All necessary precautions were maintained (i.e., remote control pipetting behind thick lead shields, etc.) in carrying out the conversions of carbonates to chlorides\*\*\*.

The  $Ca^{45}$  employed in our earliest experiments was that obtained from the Oak Ridge pile in the form of  $CaCO_3$  (AEC Catalog Item # 13 A). Since this material contained  $A^{37}$  in addition to  $Ca^{45}$ , it was deemed necessary to pump out the  $A^{37}$  under high vacuum before carrying out the conversion of the carbonate to chloride. In general, the latter conversion was carried out in a manner similar to that for  $Na^{24}$  and  $K^{12}$  above. In later experiments, high specific activity  $Ca^{45}$  was employed

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(AEC Catalog Item # S-5)\*. Aliquots of the Ca<sup>45</sup> solution were pipetted into the appropriate volumes of Ca-free artificial sea water to give the correct Ca concentration (0.012 M).

Preparation of biological material. The last stellar nerves (containing a giant axon) were excised from specimens of Loligo peallii, after first tying both ends of the portion desired. Nerve sections were then kept in fresh natural sea water for 1½ to 2 hours before use. The results of Steinbach and Spiegelman¹ had indicated that during the first 2 hours after excision of stellar nerves, the chemically determined values for Na vary considerably and it is only after this time has elapsed that the axoplasm comes into equilibrium with its outer environment. The value for Na reaches its maximum value of 10 meq. per cent within this period.

The nerves were then exposed to artificial sea water prepared according to Pantin² in which all or part of a given ion species had been replaced in isomolar concentration with radioactive material. The sea water contained 0.52 M NaCl, 0.013 M KCl, 0.012 M CaCl₂, and 0.024 M MgCl₂. The pH was adjusted to 7.7–8.0 by the addition of a small volume of bicarbonate or NaOH, the latter in those cases where the adjustment required considerable amounts of alkali. After the desired period of exposure, the nerves were removed and rinsed several times in a few changes of fresh natural sea water. After blotting of filter paper, the proximal end was cut off. The axoplasm (nerve cytoplasm) was extruded by the application of gentle but gradually increasing pressure with a pair of forceps in the direction of the cut end. The extruded axoplasm was collected on a tared aluminum planchet (130–150 mg each and about one inch in diameter) and weighed quickly with a torsion balance. One ml of distilled water was then added to each planchet to insure even distribution of the radioactive substance over the entire area of the planchet.

Determination of radioactivity. Samples were then evaporated to dryness under infra-red lamps and the radioactivity measured with a Tracerlab 64 Scaler\*\*. Measured radioactivities were recalculated to zero time from the decay curve of the individual ion under investigation in order to correct for the decomposition which occurred during the measurement of sample activities. This correction becomes appreciably large, when using Na<sup>24</sup> and K<sup>42</sup> which have half-lives of 14.8 and 12.4 hours respectively. Comparison of the activities of the samples with standards prepared from aliquots of the radioactive artificial sea water (and analysed at the same level in the counting chamber) enabled the calculation of the ion content of the axoplasm samples.

The method of preparation of the standards for Tables I, II, and III are given at the top of each of these tables. The Na standards for the data given in Tables IV, VI, IX, and X were prepared by diluting the sea water (containing 0.39 M Na<sup>23</sup>Cl + 0.13 M Na<sup>24</sup>Cl) 250 times with distilled water. 0.5 ml aliquots were then evaporated to dryness in duplicate on aluminum planchets (1.04 micromoles Na/0.5 ml). For Tables VII and VIII, Na standards were prepared by this same method. However, since a reduction in the total NaCl concentration had been made in order to maintain the isotonicity in the presence of added inhibitors of cholinesterase, the 0.5 ml aliquots contained only 1.00 micromole Na/0.5 ml. The K standards for the data given in Tables V and VII were prepared by diluting the sea water (containing 0.013 M K<sup>12</sup>Cl) 100 times and then evaporating 1.0 ml aliquots in duplicate as above (0.13 micromole K/1.0 ml). Radioactivities recorded in Tables IV through X have all been corrected to zero time.

Electrical. Nerves were tested for normality of conduction both before and after exposure to radioisotope containing sea water. The nerves were stimulated through a pair of silver wire electrodes by condenser discharge shocks of a time constant less than 0.2 milliseconds. Action potentials were led off by means of a second pair of silver wire electrodes to a condenser coupled amplifier of a modified Toeney differential type circuit and then recorded on a DuMont No. 279 Dual Beam Oscilloscope. Only those nerves were used which still exhibited normal conduction at the end of the experiment.

Studies of the rates of ion exchange during electrical activity of the nerves were carried out in the following manner: Nerve chambers were used of narrow bore polystyrene tubing (2 mm i.d.) into which were sealed, at right angles to the length and at 5 mm intervals, 0.0156" diameter Pt wire as described previously (II). Nerves were mounted in the chamber by threading a long thin wire through the polystyrene tube (one end of the wire having previously been tied to the thread attached to the nerve). The nerve was then carefully drawn into the tube. By slipping a piece of narrow bore rubber tubing over that end of the polystyrene tube from which the thread issued, the thread—and thereby the nerve—was fixed in position. The rubber tubing was then connected to a perfusion bottle filled with sea water containing the radioactive ions. Perfusion of the nerve preparation was carried out by means of gravity. The diameter of the plastic tubing chosen was such that only a very thin layer of excessive shunting by the sea water was largely eliminated and stimulation of, and recording from, the nerve was possible throughout the period of exposure to the isotope containing sea water.

 $<sup>^\</sup>star$  We are indebted to Dr G. Failla and Dr P. Aebersold for making the high specific activity Ca  $^{15}$  (carrier free) available to us.

 $<sup>\</sup>star\star$  We are indebted to Dr G. Failla and the Marine Biological Laboratory, Woods Hole, Mass., for making the Scaler available to us.

#### RESULTS

#### A. ION EXCHANGES AT REST

I. Potassium. In one series of experiments the stellar nerves were exposed to artifical sea water in which the K³9 had been replaced by K⁴2 in the usual sea water concentration (0.013 M). Analysis of axoplasm samples indicated that there was a rapid exchange of potassium under these conditions. Table I gives a few examples illustrating the size of the axoplasm samples, the magnitude of the radiation measured and the manner in which the standards were prepared. All of the data obtained in this way are presented in Fig. I. Each point on the graph represents a single experiment. The number of millimoles (mM) of K⁴² which penetrated per 100 gm axoplasm (wet weight) is plotted against time of exposure of the nerve fibre to the radioisotopic sea water. It will be noted from Fig. I that the rate of penetration of K⁴² through the nerve membrane is initially quite high but it then slows markedly and within 60 min, analyses indicate an

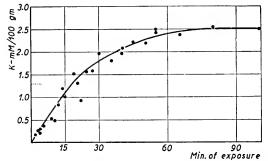


Fig. 1. K penetration across the membrane of the giant axon of Squid when exposed to 0.013 K<sup>42</sup>Cl in artificial sea water. The horizontal broken line on the ordinate indicates the K<sup>42</sup> concentration outside. The penetration of K<sup>42</sup> in millimoles (mM)/100 g axoplasm (wet weight) is plotted against time in minutes.

approach to a maximal value or 2.5 millimoles/100 g asymptotically. If one accepts the values for the potassium content of the axoplasm found in the literature (STEINBACH AND SPIEGELMAN, 32.1 meq. per cent<sup>1</sup>; BAER AND SCHMITT, 27 meq. per cent<sup>3</sup>; WEBB AND YOUNG 25.3 meq. per cent<sup>4</sup>) it can be seen that the maximum exchange obtainable under these conditions is approximately one tenth of the total K concentration of the axoplasm.

In all probability, the curve in Fig. 1 is a composite of at least two, or possibly more, distinct reactions. The first part of the curve, with the steepest slope, is, in all probability, a true measure of the

rate of exchange of K across the nerve membrane. The second phase in which the rate of exchange has slowed down may possibly be ascribed to a movement of the radioactive ions from the inside to the outside after having reached a certain level. Finally, when the inside concentration is about twice that of the outside, there appears to be an equilibrium of the movements in the two directions.

The experiments show that even at rest, there is a dynamic equilibrium between the K inside the fibre and that in its outer environment<sup>4a</sup>. Within 50 min an equilibrium is established. Under such conditions only about one tenth of the total K inside the fibre has exchanged for K<sup>42</sup> in the bathing medium. The K<sup>42</sup> concentration inside the fibre is 2.5 millimoles/100 g axoplasm against 1.3 millimoles/100 ml for the sea water. When a steady state of exchange has been attained, it is possible to calculate the permeability constant for this exchange of K at rest by means of Collander's equation as modified by Krogh<sup>5</sup>. According to Krogh where d is the diameter of the cell (cm), t is

$$P = 0.576 \frac{d}{t} \log_{10} \frac{C_s}{C_s - C_o \frac{a_s}{a_o}}$$

time (hours),  $C_s$  and  $C_o$  concentrations of the ion inside and outside respectively, and  $a_s$  and  $a_o$  are the corresponding activities. d may be assumed to be = 0.05 cm,  $C_s$  = 0.32 M (Steinbach and Spiegelman) and  $C_o$  = 0.013 M. Substituting 40400 cts/min/ml for  $a_o$  (from Table I) and 77700 cts/min/g for  $a_s$  (from Fig. 1) when t = 0.83 h, one obtains a value of 1.25·10<sup>-3</sup> cm/h for P, the permeability constant, from the equation above.

### TABLE I K<sup>42</sup> PENETRATION

Nerves exposed to sea water containing 0.013 M  $\rm K^{42}Cl$  for varying periods of time. Standards (S<sub>1</sub> and S<sub>2</sub>): sea water diluted 1:10 and then 0.5 ml evaporated to dryness in duplicate (0.65 micromole  $\rm K^{42}/0.5$  ml). Counts per min indicate the actual count, uncorrected for time decay of radioactivity.

| Time of exposure (min) | $\begin{array}{c} { m Axoplasm} \\ { m (mg)} \end{array}$ | Counts<br>per min | Millimoles<br>per 100 g | Micromoles<br>per 100 g per min |
|------------------------|-----------------------------------------------------------|-------------------|-------------------------|---------------------------------|
| 1                      | 9.2                                                       | 90                | 0.30                    | 75                              |
| 9                      | 7.6                                                       | 135               | 0.52                    |                                 |
| 14                     | 11.7                                                      | 466               | 1.19                    | 58<br>85                        |
| 19                     | 11.6                                                      | 570               | 1.51                    | So                              |
| 2.4                    | 8.3                                                       | 430               | 1.60                    | 67                              |
| 30                     | 9.2                                                       | 570               | 1.91                    | 64                              |
| 45                     | 12.9                                                      | 930               | 2.22                    | 49                              |
| 55                     | 16.0                                                      | 1247              | 2.49                    | 45                              |
| 65                     | 13.6                                                      | 1007              | 2.38                    | 37                              |
| 80                     | 18.8                                                      | 1490              | 2.54                    | 25                              |
| S <sub>1</sub>         |                                                           | 2017 \ average    | •                       |                                 |
| $S_1 \\ S_2$           |                                                           | 2022   2020       |                         |                                 |

Fig. 2 shows the rates of exchange of K against time. It will be noted that the rate is initially high but then drops to a value which is only about one fourth of that of the

is initially high but then Grops a initial rate. The rate of penetration approaches a limiting value of 20 millimoles/ 500 g/min (or 2.5 · 10<sup>-6</sup> mole/cm<sup>2</sup>/min summing an average diameter of 500  $\mu$ ).

In a second series of experiments, the nerves were exposed to 0.026 M K<sup>42</sup> 20 Cl in the bathing sea water (twice the normal K concentration). In carrying out these experiments, a decrease in NaCl concentration was made equivalent to the increase in KCl in order to maintain the isotonicity of the sea water. The data obtained are plotted in Fig. 3.

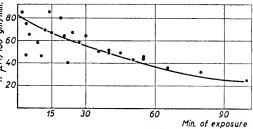


Fig. 2. Rate of K penetration across the membrane of the giant axon of Squid when exposed to 0.013 M  $\rm K^{42}Cl$  in artificial sea water. The rate of penetration of  $\rm K^{42}$  in micromoles  $(\mu \rm M)/100$  g/min is plotted against time of exposure in min.

It is evident from a comparison of Figs 1 and 3 that the shapes of the curves obtained for 0.013 M and 0.026 M KCl are very much alike. However, since the ordinate in Fig. 3 is greater by a factor of two, it can be seen that in the latter case the penetration of K<sup>42</sup> into the fibre reaches a maximal value of 5.3 millimoles/100 g axpolasm. As in the case of the experiments with 0.013 M KCl, exchange of K<sup>39</sup> inside for K<sup>42</sup> outside reaches an equilibrium when the inside concentration of K<sup>42</sup> is twice that of the outside.

As in the case of Fig. 1, Fig. 3 should probably have been resolved into three distinct phases. The considerations applied to the segments of Fig. 1 are also applicable

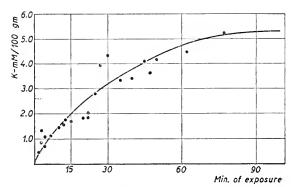


Fig. 3. K penetration across the membrane of the giant axon of Squid when exposed to 0.026 M  $\rm K^{42}Cl$  in artificial sea water (twice the normal K concentration). The horizontal broken line on the ordinate indicates the  $\rm K^{42}$  concentration outside. The penetration of  $\rm K^{42}$  in millimoles (mM/100 g axoplasm (wet weight) is plotted against time in minutes.

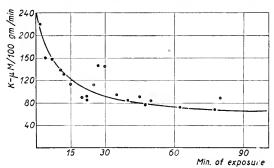


Fig. 4. Rate of K penetration across the membrane of the giant axon of Squid when exposed to 0.026 M K<sup>42</sup>Cl in the artificial sea water (twice the normal K concentration). The rate of penetration of K<sup>42</sup> in micromoles  $(\mu M)/\text{too g/min}$  is plotted against time of exposure in minutes.

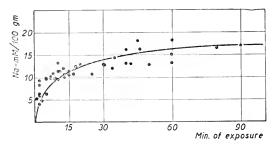


Fig. 5. Na penetration across the membrane of the giant axon of Squid when exposed to artificial sea water containing either 0.13 M or 0.065 M Na<sup>21</sup>Cl. Total NaCl concentration is 0.52 M. The penetration of Na in millimoles (mM)/100 g axoplasm (wet weight) is plotted against time of exposure in minutes.

to those of Fig. 3. The rates of K<sup>42</sup> penetration against time with 0.026 M KCl outside are given Fig. 4. From a comparison of Figs 2 and 4, it is evident that the initial rate of K<sup>42</sup> penetration, using 0.026 M KCl outside, is greater than that of the initial penetration rate obtained with 0.013 M KCl outside. Also, in the case of 0.026 M KCl outside, the rate of penetration falls more rapidly than in Fig. 1. However, the limiting rate of penetration finally attained is twice that of Fig. 2.

2. Sodium. The problem of Na penetration into the giant axons of Squid was investigated in a manner similar to that employed for K<sup>4a</sup>. In this case, however, either one fourth or one eighth of the Na23 in the sea water (normally 0.52 M) was replaced by Na<sup>24</sup>. The remainder of the Na, necessary for maintainence of isotonicity of the sea water, was made up with ordinary Na<sup>23</sup>. All other ions were maintained in their normal concentrations. Calculation of the Na penetrating the fiber was made on the assumption that there was no inherent difference in the case of Na<sup>23</sup> and Na<sup>24</sup> penetrations. Some typical data obtained are illustrated in Table II.

Fig. 5 represents all of the Na penetration data accumulated. It will be noted that Na enters the fibres at a rather high initial rate which falls markedly quite quickly. The Na penetration reaches a maximum of approximately 17.0 millimoles/100 g. This value is in good agreement with the value of 16.2 meq. per cent (16.2 millimoles/100 g) calculated by Steinbach and Spiegelman<sup>11</sup> from the data of Webb and Young. Our value for the Na penetrating would, therefore, seem to indicate that exchange of Na across the nerve membrane is

complete within about 30 min. Attainment of the steady state is accomplished when all of the Na inside the nerve has been exchanged for Na<sup>24</sup>. Under such conditions, substituting in the permeability equation, the values of 0.162 M for  $C_s$  (Webb and Young), 0.52 M for  $C_o$ , 934.3 cts/min/ $\mu$ l for  $a_o$  (Table II) and 293.6 cts/min/ $\mu$ g for  $a_s$  (Fig. 3) with t=0.5 h and d=0.05 cm, gives a value for the permeability constant of  $5.76 \cdot 10^{-2}$  cm/h.

TABLE II
Na<sup>24</sup> PENETRATION

Nerves exposed to sea water containing 0.39 M  $\rm Na^{23}Cl + 0.13~Na^{24}Cl$  for varying periods of time. Standards (S<sub>1</sub> and S<sub>2</sub>): sea water diluted 1:100 and then 0.4 ml evaporated in duplicate (2.1 micromoles/0.4 ml). Counts per min indicate the actual count, uncorrected for time decay of radioactivity.

| Time of exposure (min) | Axoplasm<br>(mg) | Counts<br>per min | Millimoles<br>per 100 g | Micromoles<br>per 100 g per min |
|------------------------|------------------|-------------------|-------------------------|---------------------------------|
| 3                      | 11.4             | 1014              | 4.7                     | 1.57                            |
| 9                      | 12.0             | 2090              | 9.7                     | 1.08                            |
| 11                     | 15.2             | 3550              | 12.4                    | 1.13                            |
| 20                     | 13.8             | 3234              | 12.8                    | 0.64                            |
| 35                     | 15.2             | 3924              | 14.3                    | 0.41                            |
| 42                     | 14.6             | 3420              | 13.0                    | 0.31                            |
| 50                     | 12.1             | 2770              | 12.7                    | 0.25                            |
| 55                     | 10.2             | 4834              | 26.8                    | 0.49                            |
| 60                     | 10.4             | 2160              | 11.7                    | 0.20                            |
| 80                     | II.I             | 3464              | 17.9                    | 0.23                            |
| $S_1 \\ S_2$           |                  | 3720 \ average    |                         |                                 |
| $S_2$                  |                  | 3754 3737         |                         |                                 |

The degree of scattering appears to be slightly larger in the case of Na than of K. This could, to some extent, be due to a slight contamination of the samples with radioactive sea water since the sea water contained such a high concentration of radioactive Na. Another factor may be the individual variations in Na content of these nerves. The data of Steinbach and Spiegelman indicate that the values vary considerably from one nerve to the next: 3 to 4 hour exposure of axons to sea water gave Na values varying from 7.8 to 17.4 meq. per cent. No apparent effort was made in their work to determine

whether or not all of these nerves maintained conduction. It is, therefore, not certain that such large deviations are actually within the normal range of variation. Nevertheless, it is quite conceivable that marked individual deviations occur.

The rates of penetration of Na into Squid nerves are plotted against time in Fig. 6. It will be noted that the initial rate of penetration of Na into fibres is extremely high but falls to a very low level within 15 to 20 min. The rate of penetration after 40 min of exposure has fallen to a value about one twen-

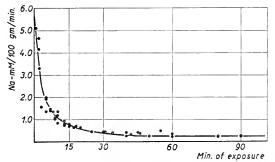


Fig. 6. Rate of Na penetration across the membrane of the giant of Squid when exposed to artificial sea water containing either 0.13 M or 0.065 M Na<sup>24</sup>Cl. Total NaCl concentration is 0.52 M. The rate of penetration of Na<sup>24</sup> in millimoles (mM)/100 g/min is plotted against time of exposure in min.

References p. 114.

tieth of that of the initial rate. This rapid fall in the rate of penetration is further support for the assumption that complete exchange of Na across the membrane occurs within a short period of time.

Extrapolation of the curve in Fig. 6 to zero time gives a value of 5.8 millimoles/100 g/min for the initial rate of Na exchange in these nerves. If one carries out a similar operation for the curve of Fig. 2, a value of 0.082 millimole/100 g/min for K is obtained. These results seem to indicate that the initial rate of exchange of Na is many times greater than of K. These findings do not support the concepts of Conway<sup>6</sup> that nerve membranes are impervious to Na although it has to be kept in mind that the observations are limited to the giant axons of Squid. The observations presented are consistent with those of Steinbach and Spiegelman who have been able to demonstrate that Na

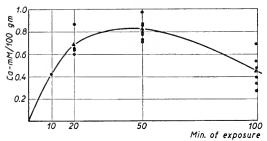


Fig. 7. Ca penetration across the membrane of the giant axon of Squid when exposed to artifical sea water containing 0.012 M Ca<sup>15</sup>Cl<sub>2</sub>. The penetration of Ca<sup>45</sup> in millimoles (mM)/100 g axoplasm (wet weight) is plotted against time in minutes.

enters these nerves.

3. Calcium. Table III gives some of the date obtained when nerves were exposed to high specific activity of Ca<sup>45</sup> (0.012 M) in artificial sea water for varying periods of time. All of the date obtained are plotted in the curve of Fig. 7. As in the cases of Na and K, each point on the curve represents a single nerve. The curve has been drawn through the mean of the several values at a given time of exposure. The data obtained were the same when low specific activity Ca<sup>45</sup> was used.

TABLE III

Ca45 PENETRATION

Nerves exposed to sea water containing 0.012 M  $\operatorname{Ca^{45}Cl_2}$  (high specific activity) for varying periods of time. Standards  $(S_1 \text{ and } S_2)$ : sea water diluted 1:200 and then 0.5 ml evaporated in duplicate (0.03 micromole  $\operatorname{Ca^{45}/0.5}$  ml).

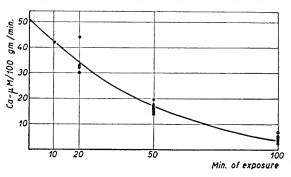
| Time of exposure (min) | Axoplasm<br>(mg) | Counts per min | Millimoles<br>per 100 g | Micromoles<br>per 100 g per min |
|------------------------|------------------|----------------|-------------------------|---------------------------------|
| . 50                   | 19.2             | 10167          | 0.79                    | 15.6                            |
| 50                     | 8.2              | 4762           | 0.87                    | 17.2                            |
| 50                     | 6.0              | 2829           | 0.71                    | 14.2                            |
| 50                     | 6.6              | 3271           | 0.74                    | 14.6                            |
| 100                    | 9.4              | 1607           | 0.26                    | 2.7                             |
| 100                    | 4.6              | 1139           | 0.37                    | 3.8                             |
| S <sub>1</sub>         |                  | 1997 \ average |                         |                                 |
| $S_2$                  |                  | 2010 2004      |                         |                                 |

It will be noted from Fig. 7 that the Ca<sup>45</sup> inside the nerve seems to reach a maximum value of 0.82 millimole/100 g within 45 min and then decreases to a value of 0.45 millimole/100 g at 100 min of exposure. It is evident, therefore, that the Ca penetrates into these nerve fibres. The values obtained seem to indicate that the concentration of Ca<sup>45</sup> at 100 min is lower than at 50 min. Further investigations are desirable for an interpretation of this observation.

Fig. 8 is a curve obtained by plotting the rates of penetration of Ca<sup>45</sup> into the nerves against time of exposure. It will be noted that the initial rate of exchange, extrapolated

to zero time, is quite high and comparable to the initial extrapolated value for K (50 micromoles/100 gm/min and 82 micromoles/100 gm/min respectively).

Fig. 8. Rate of Ca penetration across the membrane of the giant axon of Squid when exposed to artificial sea water containing 0.012 M Ca<sup>45</sup>Cl. The rate of penetration of Ca<sup>45</sup> in micromoles  $(\mu M)/100$  g/min is plotted against time of exposure in minutes.



#### B. FACTORS INFLUENCING EXCHANGE OF Na AND K

In view of the considerable individual variations of the ion content of these nerves, it appeared advisable to modify the method of accumulation of data in studying the effects of a number of factors on the ion exchanges across the nerve membrane. Instead of collecting single values at varying periods of exposure, a large number of nerves were exposed simultaneously under identical conditions and for the same period of time. At least five values were obtained for a given condition and only the average values utilized in carrying out comparisons. All exposures were limited to 30 min. They were carried out at room temperature (22° C), except for the cases in which the  $Q_{10}$  of Na and K exchange were studied.

I.  $Q_{10}$  of Na and K exchange. Table IV contains the data obtained when nerves were exposed to 0.39 M Na<sup>23</sup> Cl + 0.13 M Na<sup>24</sup> Cl in artificial sea water for 30 min at 22° and 13° C respectively. At 22° C, the average of eight nerves gave a value of 9.5 millimoles/100 g while at 13° C the average of eight nerves was 8.6 millimoles/100 g. This would correspond to a  $Q_{10}$  of 1.22.

TABLE IV EFFECT OF TEMPERATURE ON THE RATE OF PENETRATION OF Na Nerves exposed for 30 min to sea water at 22° and 13° C containing 0.39 M Na<sup>23</sup>Cl + 0.13 M Na<sup>24</sup>Cl. S<sub>1</sub> and S<sub>2</sub> = standards.

| 22° C<br>Axoplasm<br>(mg) | Counts per min            | Millimoles<br>per 100 g | 13° C<br>Axoplasm<br>(mg) | Counts<br>per min | Millimoles<br>per 100 g |
|---------------------------|---------------------------|-------------------------|---------------------------|-------------------|-------------------------|
| $S_1 \\ S_2$              | 1324 average<br>1285 1305 |                         |                           |                   |                         |
| 10.2                      | 1273                      | 9.9                     | 11.4                      | 1405              | 9.8                     |
| 9.4                       | 1321                      | 11.2                    | 16.6                      | 1964              | 9.4                     |
| 5.2                       | 590                       | 9.1                     | 14.6                      | 1624              | 8.9                     |
| 8.8                       | 996                       | 9.0                     | 20.4                      | 2100              | 8.2                     |
| 5.8                       | 599                       | 8.2                     | 12.6                      | 1199              | 7.6                     |
| 12.0                      | 1341                      | 8.9                     | 8.8                       | 1001              | 9.0                     |
| 14.2                      | 1681                      | 9.4                     | 16.6                      | 1694              | 8.1                     |
| 12.2                      | 1568                      | 10.4                    | 18.8                      | 1864              | 7.9                     |
| Average                   |                           | 9.5                     | Average                   |                   | 8.6                     |

The exchange of K was studied under identical conditions (30 min exposure at  $22^{\circ}$  and  $13^{\circ}$  C) using 0.013 M K<sup>42</sup>Cl instead of K<sup>39</sup>Cl in the sea water. At  $22^{\circ}$  C the average of seven nerves was 1.31 millimoles/100 g and at  $13^{\circ}$  C the average of the same number of nerves was 1.09 millimoles/100 g (Table V). This would correspond to a  $Q_{10}$  of 1.33.

TABLE V  ${\it Effect of Temperature on The Rate of Penetration of K} \\ {\it Nerves exposed for 30 min to sea water at 22°C and 13°C containing 0.013 M K42Cl. S_1 and S_2 = standards.}$ 

| 22° C<br>Axoplasm<br>(mg)                      | Counts<br>per min                                   | Millimoles<br>per 100 g      | 13° C<br>Axoplasm<br>(mg)     | Counts<br>per min         | Millimoles<br>per 100 g                                      |
|------------------------------------------------|-----------------------------------------------------|------------------------------|-------------------------------|---------------------------|--------------------------------------------------------------|
| $S_1$ $S_2$ 10.8 10.4 7.2                      | 1014 average<br>1051 1033<br>1230<br>1152<br>758    | 1.43<br>1.39<br>1.33         | 6.4<br>8.4<br>4.2<br>10.4     | 525<br>732<br>395<br>1030 | 1.03<br>1.10<br>1.18<br>1.25                                 |
| $S_1$ $S_2$ $7.2$ $15.8$ $12.4$ $22.0$ Average | 482 average<br>488 485<br>372<br>659<br>586<br>1000 | 1.39<br>1.12<br>1.27<br>1.22 | 6.4<br>13.2<br>9.0<br>Average | 272<br>456<br>328         | I.14<br>0.92<br>0.98<br>———————————————————————————————————— |

The values for the  $Q_{10}$  obtained above for both Na and K are in good agreement with the theoretical value of 1.25 calculated from ionic conductivity measurements. The ionic velocities increase by about 2 to 2.5% for every degree rise of temperature. It is, therefore, possible that no important energy yielding chemical reactions are involved in the exchange of ions across the nerve membrane under these experimental conditions.

2. Electrical activity and Na exchange. Stimulation of nerves by supramaximal shocks while being perfused with sea water containing 0.39 M Na<sup>23</sup>Cl + 0.13 M Na<sup>24</sup>Cl produced a marked alteration in the rate of exchange of Na when compared to resting nerves. As described under Methods, nerves were mounted in plastic chambers in which stimulating and recording electrodes were imbedded. The nerves were stimulated at a rate of 100 times per second for 30 min. Only those nerves which exhibited normal responses throughout this period of stimulation were analysed. Analysis of the axoplasm of six of these nerves indicated that 15.9 millimoles Na/100 g (mean value) had exchanged within 30 min as compared with 9.5 millimoles/100 g at rest. This would correspond to an increase in the rate of exchange of approximately 67% above that at rest. The results of the individual analyses are recorded in Table VI.

If the cation molarity (Na plus K) of the Squid axoplasm is a constant, as is suggested by the work of Steinbach and Spiegelman, then it is evident that during nerve References p. 114.

TABLE VI

EFFECT OF ELECTRICAL ACTIVITY OF THE NERVE ON THE RATE OF PENETRATION OF Na Nerves were stimulated at a rate of 100 times per second for a period of 30 min in sea water containing 0.39 M Na<sup>23</sup>Cl + 0.13 M Na<sup>24</sup>Cl at 22° C.  $S_1$  and  $S_2$  = Standards.

| Axoplasm<br>(mg) | Counts<br>per min | Millimoles<br>per 100 g |
|------------------|-------------------|-------------------------|
| S.               | 2045 ) average    |                         |
| $S_1 \\ S_2$     | 2042 2044         |                         |
| 11.2             | 3420              | 15.5                    |
| 20.4             | 6490              | 16.2                    |
| 16.0             | 5340              | 17.0                    |
| 12.2             | 4380              | 18.3                    |
| 6.2              | 1803              | 14.8                    |
| 11.4             | 3002              | 13.6                    |
| Average          |                   | 15.9                    |
| Control (see     | 9.5               |                         |

activity, a quantity of K has been lost by the nerve to the sea water equivalent to the Na which penetrated during the same period. In the case under consideration, this would be equivalent to a loss of 6.4 millimoles K/100 g of axoplasm. This loss appears to be very high since, as discussed earlier, at rest a maximum of 2.5 millimoles K/100 g are easily exchangeable.

A few calculations concerning the exchange of ions during activity of the nerve may be of interest. The average diameter of the stellar nerve may be assumed to be of the order of 500  $\mu$ . An axoplasm cylinder of r = 0.025 cm and weighing 1 g would have a surface area of 80 cm<sup>2</sup>. Since an increased exchange of 6.4 millimoles Na/100 g (or 6.4·10<sup>-5</sup> mole/g) has been demonstrated for a nerve which had been stimulated 1.8·105 times (100 per second for 30 min), it follows that 6.4·10-5 mole/g divided by 1.8·10<sup>5</sup> or 3.6·10<sup>-10</sup> mole/g/impulse of Na penetrated into the axoplasm of the nerve from the sea water. This value corresponds to 4.5 · 10<sup>-12</sup> mole of Na penetrating/cm<sup>2</sup>/impulse. It has been reported by Pumphrey and Young8 that the diameters of these giant nerve fibres of Squid usually vary from 280 to 720  $\mu$  in diameter and may in some cases by as large as 1000  $\mu$  (I mm). If one calculates the values of Na which would penetrate per cm<sup>2</sup> per impulse for the usual extremes in the size of the fibres under the above conditions, one obtains the values 2.6·10<sup>-12</sup> and 6.5·10<sup>-12</sup> mole/cm<sup>2</sup>/impulse for the smaller and larger diameters respectively. If one assumes that the increased Na penetration during activity is equivalent to the K loss during the same period, as the work of several investigators indicates, then it follows that the transfer of 4.5·10<sup>-12</sup> mole/cm<sup>2</sup>/impulse of K has occurred during the period of nerve activity. This value is in excellent agreement with that indirectly calculated by Hodgkin and Huxley<sup>9</sup> on the basis of the changes in membrane conductivity which occur in single fibre preparations of Carcinus maenus nerves during normal conduction. They obtained a value of 1.7·10<sup>-12</sup> mole/cm<sup>2</sup>/impulse. The value is also in good agreement with that obtained by Keynes<sup>10</sup>. This investigator soaked multifibre preparations of Carcinus nerves in K<sup>42</sup>. Upon stimulation he found the leakage of 2.1·10<sup>-12</sup> mole/cm<sup>2</sup>/impulse. The data with Na<sup>24</sup>, like those of Keynes, are direct. The method of Hodgkin and HUXLEY, although most ingenious, necessitates numerous assumptions and is therefore

inherently indirect. In spite of the fact that the methods and materials employed are different, the agreement is surprisingly close in the three cases.

3. Effect of inhibitors of acetylcholine-esterase on the ion exchange. The effects of two inhibitors of acetylcholine-esterase were studied on the rate of exchange of Na and K in these fibres. In Table VII are given the results obtained when giant axons were exposed for 30 min to 0.022 M diisopropyl fluorophosphate (DFP) in sea water containing 0.013 M K42Cl. DFP at this concentration is capable of abolishing nerve conduction within approximately 2 min<sup>11</sup> and the action of this compound can probably be attributed exclusively to the inactivation of the enzyme<sup>12</sup>. The average of five nerves exposed to sea water containing DFP and K42 gave a value of 1.08 millimoles K/100 g while exposure to sea water for the same period of time in the absence of DFP gave a value of 1.31 millimoles/100 g. Assuming, as above, that the average diameter of these fibres is 500  $\mu$  (area of 1 g cylinder of axoplasm being equal to 80 cm<sup>2</sup>), then one obtains a value of 5.5 · 10<sup>-9</sup> mole/cm<sup>2</sup>/min as the rate of exchange of K in sea water at rest. In the presence of DFP this rate falls to 4.5·10<sup>-9</sup> mole/cm<sup>2</sup>/min. This would correspond to a decrease of 1.0·10-9 mole/cm<sup>2</sup>/min in the presence of DFP. Although the concentration of K42 in the axoplasm is smaller in the presence of DFP than in its absence, this result does not indicate a decreased permeability. In view of the concentration gradient between the inside of the axon and its outer environment an increase in permeability may lead to an increase of the K outflow from the interior. The K42 penetrating from the outside may share the same fate and the final inside concentration will eventually be smaller than that under normal conditions.

TABLE VII  ${\rm EFFECT~of~DFP~on~The~RATE~of~PENETRATION~of~K~and~Na} \label{eq:containing} {\rm Nerves~exposed~to~0.022~M~DFP~in~sea~water~containing~either~o.013~M~K^{42}Cl~or~o.37~M~Na^{23}Cl~+~o.13~M~Na^{24}Cl.~S_1~and~S_2 = standards. }$ 

| K<br>Axoplasm<br>(mg)                                                          | Counts<br>per min                                            | Millimoles<br>per 100 g                      | Na<br>Axoplasın<br>(mg)                                                                               | Counts<br>per min        | Millimoles<br>per 100 g                                              |
|--------------------------------------------------------------------------------|--------------------------------------------------------------|----------------------------------------------|-------------------------------------------------------------------------------------------------------|--------------------------|----------------------------------------------------------------------|
| S <sub>1</sub><br>S <sub>2</sub><br>5.8<br>8.0<br>4.6<br>6.0<br>5.4<br>Average | 1014 average<br>1051 1033<br>472<br>776<br>398<br>447<br>493 | 1.02<br>1.21<br>1.09<br>0.93<br>1.15<br>1.08 | S <sub>1</sub><br>S <sub>2</sub><br>10.6<br>8.6<br>12 0<br>10.2<br>16.0<br>7.0<br>13.8<br>9.6<br>14.8 | 1324 \ average<br>1285 \ | 16.8<br>14.2<br>16.2<br>18.6<br>14.3<br>18.0<br>16.5<br>16.6<br>15.4 |
| Control (see                                                                   | e Table V)                                                   | 1.31                                         | Control (see                                                                                          | e Table IV)              | 9.5                                                                  |

This view is confirmed by the effect of the DFP on the Na movement. Table VII gives the results obtained when nerves were exposed to DFP in the same concentration as above (0.022 M) in the presence of 0.13 M  $\rm Na^{24}Cl + 0.37$  M  $\rm Na^{23}Cl$  in the sea water. The mean of nine nerves exposed to DFP in sea water gave a values of 16.4 millimoles

Na/100 g as compared to 9.5 millimoles/100 g when exposed to sea water in the absence of DFP. This would correspond to a rate of penetration of Na of  $4.0 \cdot 10^{-8} / \text{cm}^2 / \text{min}$  in the absence of DFP and a penetration of  $6.9 \cdot 10^{-8}$  mole/cm²/min in the presence of DFP, assuming the average fibre diameter to be  $500 \, \mu$ . The rate of Na penetration has increased markedly. This could be expected on the basis of the concentration gradient in the event of increased permeability. It may be noted that the Na penetration has increased to a greater extent than the K penetration has decreased. Considering the difference in the rates of entrance of Na and K, it has to be kept in mind that in the experiments described, only the penetration of ions into the interior has been determined. No measurements have been carried out in respect to the leakage of K. If the amount of K actually passing from the inside to the outside were considerably increased, this would not be indicated by the method used.

The effect of eserine, another inhibitor of acetylcholine-esterase, on the rate of Na penetration into the nerve was also studied. The results are given in Table VIII. It will be noted that 13.2 millimoles Na/100 g enter these nerves in the presence of 0.019 M eserine in the sea water containing 0.13 M Na<sup>24</sup>Cl + 0.37 M Na<sup>23</sup>Cl. This would correspond to a rate of exchange of Na of  $5.5 \cdot 10^{-8}$  mole/cm²/min in the presence of eserine as compared to  $4.0 \cdot 10^{-8}$  mole/cm²/min in its absence, again assuming the average fibre diameter to be 500  $\mu$ . The above value is the average of ten nerves and, as in the other experiments, nerves were exposed for 30 min to the eserine-containing sea water. Eserine, in the concentration used, abolishes nerve conduction reversibly within 5–15 min. The time required to abolish the action potential of these nerves shows considerable variation in the case of eserine and is closely dependent upon the  $p_H$  and other factors<sup>13</sup>. Air oxidation of the eserine proceeds rapidly at the  $p_H$  employed (7.7–8.0) and therefore

| Axoplasm          | Counts         | Millimoles |
|-------------------|----------------|------------|
| (mg)              | per min        | per 100 g  |
| $S_1$             | 2002 ) average |            |
| $S_2$             | 1910 1956      |            |
| 22.0              | 5307           | 12.3       |
| 15.0              | 3705           | 12.6       |
| 19.4              | 5550           | 14.7       |
| 3.8               | 1019           | 13.7       |
| Sı                | 1820   average |            |
| ${f S_1} {f S_2}$ | 1861 1841      |            |
| 9.2               | 2458           | 15.1       |
| 22.4              | 4950           | 12.0       |
| 8.4               | 1946           | 12.6       |
| 14.8              | 3660           | 13.4       |
| 20.8              | 4720           | 12.3       |
| 26.0              | 6490           | 13.5       |
| Average           |                | 13.2       |
| Control (see      | Table IV)      | 9.5        |

a given solution cannot be used for a prolonged period of time. The results presented were obtained with fresh eserine solutions. Although there is a marked increase in Na exchange, the effect of eserine is not as large as that obtained with DFP.

4. Cocaine and Na exchange. The effects of cocaine in 0.005 M in sea water have been studied using 0.13 M Na<sup>24</sup>Cl + 0.39 M Na<sup>23</sup>Cl in the bathing fluid. Nerves were exposed to this solution for 30 min. The results are reported in Table IX. No decrease in membrane permeability is evident from the data. The Na exchange amounted to 11.2 millimoles/100 g (average of six nerves). Again assuming a fibre diameter of 500  $\mu$ , this would correspond to a rate of Na exchange of  $4.6 \cdot 10^{-8}$  mole/cm<sup>2</sup>/min, a slight increase compared with the control.

TABLE IX EFFECT OF COCAINE ON THE RATE OF PENETRATION OF Na Nerves exposed to 0.005 M cocaine in sea water containing 0.39 M Na $^{23}$ Cl + 0.13 M Na $^{24}$ Cl. S $_1$  and S $_2$  = standards.

| Axoplasm<br>(mg)                        | Counts<br>per min | Millimoles<br>per 100 g |
|-----------------------------------------|-------------------|-------------------------|
| S *                                     | 2045 average      |                         |
| S <sub>1</sub><br>S <sub>2</sub><br>6.4 | 2042 2044         |                         |
| 6.4                                     | 1301              | 10.5                    |
| 5.8                                     | I102              | 9.9                     |
| 4.8                                     | 995               | 10.8                    |
| 12.8                                    | 2983              | I2.I                    |
| 4.8                                     | 1142              | 12.3                    |
| 7.2                                     | 1616              |                         |
| Average                                 |                   | 11.2                    |
| Control (see                            | Table IV)         | 9.5                     |

5. Effect of X-ray irradiation. The effects of high intensity X-ray irradiation on the membrane permeability to Na was studied. Nerves were irradiated with 50000 R and 125000 R while immersed in a shallow dish containing natural sea water (water layer about 5 mm thick). Immediately after irradiation, the nerves were transferred to artificial sea water containing 0.39 M Na<sup>23</sup>Cl + 0.13 M Na<sup>24</sup>Cl. After 30 min exposure to sea water the nerves were analysed. Only those nerves which still exhibited normal conduction upon stimulation were used. The results are given in Table X.

In the axoplasm of nerves irradiated with 125000 R, an average value of 14.1 millimoles/100 g was found (average of seven values). This corresponds to a penetration of 5.9·10<sup>-8</sup> mole/cm<sup>2</sup>/min. Consequently, the rate of penetration had markedly increased. The findings suggest that irradiation had strongly increased the permeability.

Irradiation with 50 000 R gave an average value of 10.9 millimoles Na/100 g (average of eight nerves). This corresponds to a rate of penetration of Na of 4.7·10<sup>-8</sup> mole/cm<sup>2</sup>/min. The increase in the rate of penetration is relatively small but appears significant, especially in connection with the high increase observed with the larger dose of irradiation. It may be noted that the effect was obtained immediately after irradiation.

| 50 000 R<br>Axoplasm<br>(mg)                                                    | Counts<br>per min                                                 | Millimoles<br>per 100 g             | 125000 R<br>Axoplasm<br>(mg)                                     | Counts<br>per min                                 | Millimoles<br>per 100 g                                  |
|---------------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------|------------------------------------------------------------------|---------------------------------------------------|----------------------------------------------------------|
| S <sub>1</sub><br>S <sub>2</sub><br>10.4<br>13.4<br>13.8                        | 2002   average<br>1910   1956<br>2425<br>2620<br>2375             | 12.4<br>10.4<br>9.1                 | 7.0<br>5.0<br>3.8<br>6.2                                         | 1610<br>1248<br>1033<br>1700                      | 12.2<br>13.3<br>14.5<br>14.6                             |
| S <sub>1</sub><br>S <sub>2</sub><br>6.6<br>7.6<br>8.4<br>9.8<br>11.8<br>Average | 1820 average<br>1861 1841<br>1198<br>1565<br>1576<br>1696<br>2725 | 10.3<br>11.6<br>10.6<br>9.7<br>13.0 | S <sub>1</sub><br>S <sub>2</sub><br>5.6<br>4.0<br>5.4<br>Average | 2045 average<br>2042 2044<br>1380<br>1225<br>1711 | 12.5<br>15.6<br>16.1<br>———————————————————————————————— |

## DISCUSSION

From the results obtained upon exposure of nerves to sea water, at rest, containing radioactive K<sup>42</sup>, it can be seen that part of the K of the nerve interior is in dynamic equilibrium with that in the outer bathing medium. The lack of exchange of approximately 90% of the K<sup>39</sup> under these conditions is unexplained. It appears that most of the K inside the nerve is not easily lost by the cell. Once the free, easily diffusible K has been exchanged for K<sup>42</sup>, the rate of K exchange falls to a very low level. This is in good agreement with the observations of Hevesy and Hahn on rabbit muscle and red blood cells<sup>14</sup>, of Steinbach on *Thyone briareus* muscle<sup>15</sup>, and of Heppel on rat muscle<sup>16</sup>. In all of these investigations no more than 10–30% of the total K content of the tissues under investigation was exchangeable at rest.

In an effort to explain the difficulty of incomplete K exchange essentially two theories have been discussed. The one considers the possibility that the K is present in bound form. The idea has been proposed that a K salt of an unknown organic acid with a very low dissociation constant exists. As emphasized by Kroch<sup>5</sup>, there is no evidence for the existence of bound K and from a theoretical basis, it appears doubtful that it can exist. Hill and Kupalov<sup>17</sup> have shown that all the K inside the muscle cell is required to be in ionic form in order to account for the osmotic pressure. Moreover, its presence in ionic form is necessary to insure the neutral reaction. Another possibility discussed is the presence of K impermeable barriers inside the cell. No such structures are known. The reasons for exchange of only a small fraction of the total K cannot be resolved at present.

The values for the  $Q_{10}$  for K and Na exchange obtained, I.22 and I.33 respectively, are in good agreement with the value of I.25 calculated theoretically from ionic conductively measurements. These figures do not support the assumption that important energy yielding reactions are involved in the transport of ions across these nerve membranes in resting condition. Krogh discusses the possibility that the extrusion of Na from the cell interior is an active process requiring energy. In support of this hypothesis, he cites experiments of Harris<sup>18</sup> and Danowski<sup>19</sup> with rabbit and human erythrocytes in which it had been shown that, at low temperature and at body temperature in the absence of glucose, K is lost to the bathing medium and replaced by Na. When glycolysis is restored, the normal K balance is restablished, even *in vitro*, with a resumption of rapid Na extrusion. If the extrusion of Na is an active process in the nerve preparation tested, under resting condition, one would have expected to obtain a larger value for the  $Q_{10}$ . Lowering the temperature of these nerves by ten degrees should have produced a marked effect on the glycolytic processes and should have been expected to yield larger Na values than those obtained.

The fact that in resting condition no expenditure of energy seems to be required for the ionic movements does by no means preclude the possibility that under other conditions these movements may require energy. It appears likely that during the early growth stage of these nerves chemical reactions are in operation which are responsible for the establishment of the large concentration gradient between the potassium inside the fibre and that in the outer bathing fluid. The same is true for the disequilibrium observed after activity. The extra oxygen uptake observed after activity indicates that energy yielding reactions are involved in the restoration of the resting condition.

The present studies of the ion exchange occurring in nerve during activity have indicated that the Na content increases markedly. Similar results have been obtained with muscle tissue by Fenn et al. on frog, and rat<sup>20, 21, 22</sup>, Wood, Collins and Moe on dog gastrocnemius<sup>23</sup>, Tipton on cat muscle<sup>24</sup>, Heppel on K-deprived rats<sup>25</sup> and Hahn and Hevesy on rats<sup>14</sup>. All of these investigations show that in contracting muscles the permeability to ions is increased. K is lost from the fibres and is replaced by Na. Steinbach and Spiegelman<sup>1</sup> have demonstrated that the cation molarity of the Squid axoplasm is, under a variety of conditions, constant at rest. It appears, therefore, justifiable to assume that during nerve activity K loss is compensated for by the penetration of an equivalent quantity of Na into these fibres.

This idea is supported by the demonstration of the penetration of 4.5·10<sup>-12</sup> mole Na/cm²/impulse, a value which is in close agreement with the value of 1.7·10<sup>-12</sup> mole K/cm²/impulse found by Hodgkin and Huxley³ and 2.1·10<sup>-12</sup> mole K/cm²/impulse reported by Keynes¹³. The value reported here indicates that during activity a considerable increase of Na inside takes place. 6.4 millimoles per 100 g were found after 30 min stimulation at 100 per second as compared with 1.3 millimoles per 100 g at rest. If an equivalent amount of K has leaked out, 21% of the total K content has been exchanged during this stimulation period. It should be noted here that the period of stimulation employed is by no means the maximum possible with these nerves. Much more prolonged periods of stimulation at 100 per second are possible and one would expect an even greater ion exchange. It should be borne in mind that the above changes are completely reversible and cessation of stimulation should result in restoration of the normal balance. From the above considerations, it may be concluded that, even though 90% of the K content of the nerve is not exchangeable at rest, during activity

some reactions have occurred which facilitate the more rapid loss of K by these fibres.

A short discussion of the methods employed in the papers of Hodgkin and Hunley and Keynes as compared with the present investigations might be of interest. The method used by Hodgkin and Hunley involves measurement of the small changes in the ionic conductivities over small areas of the nerve membranes before and after activity. Both the electrical recording equipment and the electrode assemblies are complex and the method employed necessitates numerous assumptions. The method employed by Keynes is more direct. However, he has used multifibre preparations. Under such circumstances, one could expect a retarded diffusion of K42 away from the nerve preparation because of the possible trapping of K in the intracellular fluids. Since only the radioactivity of the K42 remaining in the nerve preparation was measured in these investigations, one would expect that values obtained in this manner would be higher than the actual intracellular K42 content of the fibres. The calculated value for the K leakage per cm2 per impulse would therefore be expected to be smaller than the true value.

The method employed in the present investigation is direct. Since it is possible to analyse directly the axoplasm of the single nerve fibre, the values obtained must be considered to be more precise than those obtained by either of the above methods. The only assumption involved is the exact size of the individual fibres employed. However, since all of the Squid used were of approximately the same size, it is safe to assume that the fibres were all of approximately the same diameters. For medium size Squid this is approximately 500  $\mu$  (0.05 cm). It is justifiable to assume that the average value is close to this figure.

The investigation of the effect of inhibitors of acetylcholine-esterase on the rates of the ion exchange across the nerve membrane requires some comment. It has been shown that exposure of nerves to sea water for 30 minutes containing K42 plus DFP causes a decrease in the rate of K exchange from 1.31 to 1.08 millimoles per 100 g. The exposure of nerves to DFP has apparently altered the permeability of the nerve membrane. The DFP could conceivably have affected the membrane by decreasing its permeability. However, the effect of DFP on the rate of Na penetration excludes this interpretation. The value for the Na penetration markedly increased from 9.6 millimoles Na per 100 g to 16.4 millimoles upon the addition of 0.022 M DFP. If the DFP had had the effect of decreasing the membrane permeability one would have expected a decreased Na exchange. It might have been expected that with increased ion permeability the K could penetrate into the fibre more readily, However, since the concentration of K inside of these nerves is approximately 20 times that of sea water, it is likely that the easily exchangeable K will rapidly diffuse out into the sea water in an attempt to equalize the adverse concentration gradient across the nerve membrane. The K, in this case, will be replaced by the entrance of Na in order to maintain the electrical neutrality of the axoplasm. In such an event, the exchange of K42 would proceed at a decreased rate and this obviously accounts for the decreased K exchange in the presence of DFP. Thus, the Na and K exchange measurements are consistent with the concept that the membrane permeability had been increased by the DFP.

The probability of the exchange of  $K^{39}$  for radioactive  $Na^{24}$  was discussed before. Another factor to be considered is the constancy of the total cation content of these nerves. It has been demonstrated by Steinbach and Spiegelman¹ that under normal resting conditions the cation content (Na + K) of these nerves is a constant. However,

it is not known whether nerves in which the permeability has been increased still maintain their normal total cation concentration. It is possible that under these conditions Na as well as Cl may diffuse into the cell. This would result in increased total base content. Since the total base content of the axoplasm samples has not been measured, the contribution by the NaCl diffusion into the nerve cannot be evaluated. This problem has to be investigated further.

The effect of eserine, another inhibitor of acetylcholine-esterase had a similar but less marked effect than DFP in increasing the membrane permeability to Na. It may be noted, that in the case of DFP conduction was, on the basis of previous experience, abolished irreversibly. In the case of eserine the effect was almost certainly still reversible.

The result obtained with acetylcholine-esterase inhibitors, suggest that these substances may be capable of altering the membrane permeability. Since the only known action of these compounds is the inhibition of the enzyme acetylcholine-esterase<sup>26</sup> which is known to be closely connected with nerve conduction, it is possible that the effect observed is a manifestation of the inactivation of the enzyme. These experiments do not permit any definite conclusion, especially in view of the irreversible action of DFP during the long exposure period used. However, they may open a new approach to the importance of the acetylcholine-esterase system in the permeability of the surface membrane to ions.

The study of effects of cocaine on the membrane permeability to Na has indicated a small increase in the rate of exchange. The data are inadequate to judge whether or not this increase is significant. Employing the same concentration of cocaine (5·10<sup>-3</sup> M), Shanes<sup>27</sup>, from membrane potential measurements, came to the conclusion that a decrease in permeability had been accomplished. The results obtained here fail to confirm his reports.

The study of effects of irradiation of nerves with large doses of X-rays (50000 R and 125000 R) indicates that immediately following exposure, marked alterations in membrane permeability are evident. Exposure to 125000 R caused a large increase in membrane permeability while 50000 R caused only a small but significant increase. It should be noted that these studies were carried out immediately after irradiation. It is possible that a more marked effect would be evident with smaller doses of irradiation if longer periods of time were permitted to elapse between irradiation and exposure to radioactive ions. From our present knowledge, it is clear that the most notable effects of exposure to radiation occur after prolonged periods of time so that a longer time lapse than that used in these experiments might be preferable. It appears significant that it has been possible to demonstrate increased membrane permeability as result of X-ray irradiation.

I wish to express my gratitude to Dr David Nachmansohn for suggesting these investigations and for the guidance and encouragement he has given throughout the course of this research. I am indebted to Mrs Emily Feld-Hedal and Mrs Heidi Richards for their assistance in the experiments.

### SUMMARY

<sup>1.</sup> Studies on the permeability of the surface membranes of the giant axon of Squid to K indicate that a dynamic rather than a static equilibrium exists at rest. Approximately 10% of the total K References p. 114.

in the fibre is replaced by  $K^{42}$  from the bathing medium within one hour. When the nerve is bathed in twice the normal K concentration (0.026 M) the K content of the axoplasm reaches a maximum twice that obtained with the normal K concentration outside.

2. Exposure of nerves to sea water containing Na<sup>24</sup> results in a total exchange of all of the Na in the axoplasm for its radioactive isotope within 20 to 30 minutes.

3. Studies with Ca<sup>45</sup> in the outer bathing fluid indicate an uptake of Ca<sup>45</sup> to the extent of 0.85 millimoles per 100 g within 45 minutes and then a decrease to 0.45 millimoles per 100 g at 100 minutes of exposure.

4. The temperature coefficient  $(Q_{10})$  obtained from the rates of exchange of Na and K does not indicate that there are important energy yielding chemical reactions involved in the exchange of ions across the membrane at rest. The values obtained (1.22 for K and 1.33 for Na) are in good agreement with the theoretical value (1.25) calculated from ionic conductivity measurements.

5. Electrical activity causes an increased rate of Na penetration into the fibre. 4.5·10<sup>-12</sup> mole of Na enter per cm<sup>2</sup> per impulse.

6. Inhibitors of cholinesterase, e.g., eserine and DFP, seem to produce an increase in membrane permeability. The rate of  $K^{42}$  penetration is decreased, that of  $Na^{24}$  increased.

7. Exposure to cocaine (0.005 M) does not affect markedly the rate of Na<sup>24</sup> penetration.

8. X-ray irradiation with 125000 R produces a large and immediate increase in membrane permeability to Na<sup>24</sup> whereas 50000 R produces a smaller effect but in the same direction.

#### RÉSUMÉ

- I. L'étude de la perméabilité au potassium de la membrane du cordon nerveux principal de Seiche indique l'existence au repos d'un équilibre dynamique plutôt que statique. Environ le 10% du K total de la fibre est remplacé par K<sup>42</sup> du milieu environnant en une heure. Si le nerf est immergé dans une solution de concentration de K deux fois plus grande que la concentration normale (0.026 M) la teneur en K de l'axoplasme atteint un maximum qui est égal au double de la valeur obtenue avec une concentration externe normale de K.
- 2. Si l'on expose un nerf à l'eau de mer contenant  $Na^{24}$  un échange total a lieu entre le Na de l'axoplasme et son isotope radioactif en 20 à 30 minutes.
- 3. Si le bain extérieur contient Ca<sup>45</sup>, celui-ci est absorbé jusqu'à 0.85 millimoles par 100 g en 45 minutes, puis la concentration de Ca<sup>45</sup> décroît jusqu'à une valeur de 0.45 millimoles par 100 g au bout de 100 minutes.
- 4. Le coefficient de température  $(Q_{10})$  obtenu à partir des vitesses d'échange de Na et K ne semble pas indiquer que des réactions chimiques dégageant d'importantes quantités d'énergie soient liées à l'échange des ions à travers la membrane. Ses valeurs obtenues (1.22 pour le K et 1.33 pour le Na) sont en accord avec la valeur théorique (1.25) calculée à partir de mesures de conductivité ionique.
- 5. L'activité électrique augmente la vitesse de pénétration du Na dans la fibre.  $4.5 \cdot 10^{-12}$  mols de Na pénétrent par cm² et par influx.
- 6. Les inhibiteurs de l'acétylcholine estérase, p. ex. l'ésérine et le DFP semblent, augmenter la perméabilité de la membrane. La vitesse de pénétration de K<sup>42</sup> diminue tandis que celle de Na<sup>24</sup> augmente.
- 7. Une exposition à la cocaı̈ne (0.005 M) n'affecte pas considérablement la vitesse de pénétration de  $\mathrm{Na}^{24}$ .
- 8. L'irradiation aux rayons-X de 125000 R produit une augmentation importante et immédiate de la perméabilité de la membrane au Na<sup>24</sup>. 50000 R produisent un effet moindre dans le même sens.

# ZUSAMMENFASSUNG

- 1. Die Permeabilität der Membranen des Hauptnervenstranges vom Tintenfisch (*Loligo peallii*) für K wurde untersucht und gefunden, dass in der Ruhe eher ein dynamisches als ein statisches Gleichgewicht zu bestehen scheint. Ungefähr 10% des gesamten K-Gehaltes der Faser werden innerhalb einer Stunde durch K<sup>42</sup> aus der umgebenden Lösung ersetzt. Ist der K-Gehalt des Bades zweimal so gross wie die normale Konzentration (0.026 M), dann ist auch der maximale K-Gehalt des Nervenstranggewebes zweimal so gross wie bei normaler äusserer Konzentration.
- 2. In Na<sup>24</sup>-haltigem Meerwasser findet ein vollkommener Austausch des im Gewebe enthaltenen Na gegen sein radioaktives Isotop innerhalb 20 bis 30 Minutes statt.
- 3. Enthält das äussere Bad  $\overline{\text{Ca}}^{45}$ , so wird dieses bis zu 0.84 Millimol per 100 g in 45 Minuten aufgenommen; dann nimmt der  $\overline{\text{Ca}}^{45}$ -Gehalt wieder ab und beträgt noch 100 Minuten 0.45 Millimol per 100 g.
  - 4. Der aus den Austauschgeschwindigkeiten für Na und K errechnete Temperaturkoeffizient

 $(Q_{10})$  weist nicht darauf hin, dass in der Ruhe stark exothermische chemische Reaktionen an dem Ionenaustausch durch die Membrane beteiligt sind. Die erhaltenen Werte (1.22 für K und 1.33 für Na) stimmen gut mit dem aus Messungen der Ionenleitfähigkeit errechneten theoretischen Werte (1.25) überein.

5. Durch elektrische Arbeit wird das Eindringen von Na beschleunigt. 4.5·10<sup>-12</sup> Mol Na per cm²

dringen bei jeder Anregung ein.

- 6. Hemmstoffe der Acetylcholinesterase, wie Eserin und DFP scheinen die Permeabilität der Membrane zu erhöhen. K<sup>42</sup> wird langsamer, Na<sup>24</sup> rascher aufgenommen.
  - 7. Cocaïn (0.005 M) beeinflusst die Aufnahmegeschwindigkeit von Na<sup>24</sup> nicht merklich.
- 8. Bestrahlung mit Röntgen-Strahlen (125000) erhöht R die Permeabilität für Na<sup>24</sup> augenblicklich stark, mit 50000 R ist dieser Effekt gleichgerichtet aber geringer.

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# NERVE CONDUCTION WITHOUT INCREASED OXYGEN CONSUMPTION; THE ACTION OF AZIDE AND FLUOROACETATE\*

by

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The precise correlation of an extra oxygen consumption of active nerve with an extra heat production was established nearly a quarter of a century ago by one of us in Prof. Meyerhof's laboratory. It is an especial pleasure to report the present extension of such studies, in his honour. Nor can we refrain from an expression of admiration for his continued vigour of thought and research despite a weight of personal disaster that would have crushed most men.

That the extra energy release of nerve activity is essential to conduction and recovery was taken for granted since its discovery. With energy sources blocked by oxygen lack or IAA poisoning, conduction failed. With tetanization at a rate to limit full development of the delayed heat and oxygen consumption, conduction was depressed. Restoration of full metabolism restored full conduction in all cases. The actual fuel burned proved not identical for rest and activity. True, both resting and active metabolism seemed to focus on the production of energy-rich phosphate bonds, especially as creatine phosphate. And true, also, that the procedures that blocked conduction affected resting as well as active respiration. Nonetheless, there seemed no reason to question the essential contribution of the active respiration to actual conduction. A tentative report by Schmitt, of a fall in oxygen consumption on stimulation of yohimbinized nerve, was given little weight; and Lorente de No's finding, that excitation could be restored in a nerve blocked by anoxia, with the aid of a repolarizing current, did not really question the necessity of the metabolism as a normal source of membrane polarization.

Yet it was early shown by Feng and in this laboratory that lactate, indifferent to nerve conduction and metabolism under normal conditions, could restore resting oxygen consumption and active conduction after IAA poisoning — suggesting some interchangeability of resting and active metabolic energy. Further, 90 to 97% of the energy of activity is liberated after an impulse has traveled and the nerve again reset for action. Moreover, a factor of safety of five for the resting metabolism could be estimated. Activity might, then, be supported under emergency conditions by a portion of the resting metabolism. Sodium azide, found by Stannard to eliminate the contraction respiration of muscle, was tested on nerve in Bronk's laboratory and here and found indeed able to abolish the extra oxygen consumption of active nerve while leaving conduction intact and resting respiration largely so. We found, further, that methyl

<sup>\*</sup> This work was performed under contract with the Office of Naval Research.

fluoroacetate can reduce the resting oxygen consumption below half normal while leaving conduction and the attendant respiration increase intact. Resting and active respiration are thus sharply separable, yet they are effectively interchangeable in support of function.

For these studies, a modified Gerard-Hartline capillary respirometer was developed. Ten slots in a plexiglass block served as nerve chambers, each fitted with stimulating and lead-off electrodes. Capillaries led from each into a large chamber machined in the same block, the whole being covered with a plexiglass sheet and mounted in a glass-walled water bath. The movement of dodecane indicator drops in the capillaries was followed with a horizontal microscope mounted on the compound rest of an 11 inch lathe. Stimuli at 120/sec gave an action spike of about 25 mm measured on the cathode ray tube face.

The resting  $Q_{0_2}$  of twenty four pairs of frog sciatics at 24° C (22 to 26) centered around 65 and the two nerves of a pair agreed within 12% (aver. 4%) in all but three cases. The increased  $Q_{0_2}$  on maximal stimulation averaged 21, but with an average difference between members of a pair of nearly 30%. The coefficient of correlation between spike height and activity  $Q_{0_2}$  was only 0.4 for 67 normal nerves, and that between resting  $Q_{0_2}$  and the active increase, —0.1. Even allowing for methodological errors, these data suggest some real independence of the three variables.

In ten experiments with Na azide (0.1 or 0.3 mM,  $p_H$  7.5, 1 hour soak), spike height of the exposed nerves averaged 88% of their undrugged partners, while the  $Q_{02}$  increase on tetanization was only 12% of the normals. In four experiments with spike height in both nerves of a pair alike, the  $Q_{02}$  increase in the azide member was 0 or 1. Even these azide concentrations do not fully spare the resting metabolism, which was depressed by 0 in 4 experiments to some 50% in 2. When resting oxygen was cut in two and the active increase abolished, spike height was greatly reduced. Stronger azide (5 or 10 mM) cut resting  $Q_{02}$  to 20–35% of normal and stopped conduction. Full conduction without increased  $Q_{02}$  is possible for at least 4 hours.

In 11 experiments with MFA (1 to 2.5 mM), the spike height and the extra  $Q_{02}$  of activity remained entirely normal in the exposed nerves, while the resting  $Q_{02}$  was depressed 25% on the average, one third maximum. This depression cannot be solely of non-axonal tissue (e.g., Schwann cells), for fiber thresholds rise acutely. With stronger MFA (13 experiments at 5 or 7.5 mM), resting and active  $Q_{02}$  were both cut to about half and spike height to under two-thirds normal. In individual cases, the active spike and  $Q_{02}$  were essentially normal with resting  $Q_{02}$  depressed to one-third; in one case activity responses remained normal for 7 hours with resting  $Q_{02}$  at 50%. More usually with resting  $Q_{02}$  cut in half the active increase was also abolished while spike height remained close to normal.

A nerve can thus continue to conduct for hours with no increase in oxygen consumption and even with some half its resting respiration lost. Whether other energy sources are being tapped or even whether the small initial heat persists without delayed heat under such drug action, could be determined by heat measurements; but it seems most likely that the extra energy for activity is somehow derived from the resting metabolism by virtue of the considerable safety factor normally present.

### SUMMARY

Using a modified Gerard-Hartline capillary respirameter the resting respiration of frog nerve at  $24^{\circ}$  C was measured,  $Qo_2$  65, as well as the increase on tetanization at 120/sec,  $Qo_2$  21, and the

action spike. Azide (0.1-0.3 mM) can abolish the activity increase of oxygen consumption while leaving intact (sometimes) the resting level and conduction. Methylfluoroacetate (2 mM), conversely, can reduce the resting oxygen consumption below half while leaving intact the activity increase and conduction. Resting and active metabolism are thus separable and conduction can continue at least seven hours with no extra respiration and even with half depression of the resting level.

#### RÉSUMÉ

Au moyen d'un respiromètre capillaire Gerard-Hartline modifié, on a mesuré à 24° la respiration de nerfs de grenouille au repos (Qo<sub>2</sub> 65), son augmentation par tétanisation à 120/sec (Qo<sub>2</sub> 21), et la "pointe" d'action. L'ion  $N_3$  (0.1–0.3 mM) peut abolir l'accroissement de consommation d'oxygène dû à l'activité, tout en laissant intacts (parfois) le niveau du repos et la conduction. Le fluoracétate de méthyle (2 mM) par contre peut réduire la consommation d'oxygène au repos de plus de la moitié tout en laissant intacts l'accroissement dû à l'activité et la conduction. Le métabolisme au repos et pendant l'activité sont ainsi séparables, et la conduction peut continuer pendant au moins 7 heures sans respiration supplémentaire et même avec un abaissement de moitié du niveau du repos.

#### ZUSAMMENFASSUNG

Mittels eines abgeänderten Gerard-Hartline Kapillar-Respirometers wurde die Atmung des ruhenden Frischnervs bei 24° gemessen (Qo<sub>2</sub> 65), desgleichen die Steigerung durch Tetanisierung bei 120/sek. (Qo<sub>2</sub> 21) und die "Wirkungspitze". Azid (0.1–0.3 mM) kann die Steigerung des Sauerstoffverbrauchs bei der Arbeit unterdrücken, während der Verbrauchsspiegel bei Ruhe (manchmal) und die Übertragung unverändert bleiben. Methyl-fluoracetat (2 mM) dagegen kann den Sauerstoffverbrauch bei Ruhe unter die Hälfte herabdrücken, während die Steigerung bei Arbeit und die Übertragung unberührt bleiben. Ruheumsatz und Arbeitsumsatz sind also trennbar, und die Übertragung kann mindestens 7 Stunden lang fortbestehen ohne zusätzliche Atmung, und sogar mit einem auf die Hälfte herabgeminderten Ruhespiegel.

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# SOME EVIDENCE ON THE FUNCTIONAL ORGANIZATION OF THE BRAIN

by

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"With health, the assertion is that each person's normal thought and conduct are, or signify, survivals of the fittest states of what we may call the topmost "layers". Now suppose that from disease the normal highest level of evolution (the topmost layer) is rendered functionless. This is the dissolution . . . I contend that his mental symptoms are survivals on the lower, but then highest, level of evolution" (remaining in function).

So wrote Hughlings Jackson in 1884. One type of evidence for such an evolutionary concept involving a hierarchy of levels is observed by studying behaviour following a series of surgical sections of the brain. A transection below the medulla gives rise to the spinal animal<sup>2</sup>, a decapitated preparation kept alive by artificial respiration but still responding to stimulation with primitive though appropriate muscular actions. A painful stimulus applied to the foot pad, for example, evokes flexion of that leg, a movement that makes for survival as the leg is withdrawn from harm.

The decerebrate animal produced by cutting through a higher level<sup>2</sup>, namely the lower portion of the midbrain and therefore retaining the medulla reveals a release of the antigravity muscles permitting an abnormal sort of erect standing called decerebrate rigidity. The decorticate animal with extirpation of the highest portion of his brain only, expresses sham rage, a release of emotional patterns from cortical control<sup>3</sup>. Both decerebrate rigidity and sham rage may appear spontaneously or may be evoked. These three sections of the neuraxis reveal patterns of behaviour which are functional in the intact organism but are modified by anatomically higher areas, of later development which facilitate more delicate sensory perception and finer execution of movement. For the organism to take advantage of these improved capacities the behaviour of the lower portions of the brain must be subjected to the inhibition as well as the reinforcement of the higher planes and when their influence is removed we see a release of function in the lower areas, a result of loss of restraint. Strong support for the observation that inhibition is a function of the brain has been afforded by the physiological experiments of Dusser de Barenne and McCulloch4 who demonstrated that stimulation of one cerebral area suppresses activity in another.

For another type of evidence we must turn to an examination of man for an opportunity is afforded to study the human brain when sections are made in a functional manner. An example is observed during hypoglycemia when a temporary "dissolution" of the brain is a result of excessive insulin<sup>5</sup>. The behavioural phenomena observed may be allocated to certain cerebral areas. In fact, the signs exhibited are those that might

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be expected if successive surgical sections were made at different levels of the brain.

In order to explain the changes observed in hypoglycemia it must be recalled that glucose is no longer available to the brain. Since glucose is the chief foodstuff of the brain<sup>6, 7, 8</sup> the metabolic fires falter because of the decrease in the coal to be burned<sup>9</sup>. A decrease to 52 %<sup>10</sup> and 40 %<sup>11</sup> respectively of the normal rate have been reported in hypoglycemia. With the most profound metabolic depression (*i.e.*, in the 5th phase, see below) cerebral metabolic rate may be reduced to 25 % of the normal<sup>11</sup>. But not all parts of the brain are effected to an equal degree. Though the brain possesses a high rate of metabolism, the rate is not the same in all regions but in general exhibits a quantitative gradient along the neuraxis, most intense anteriorally and superiorally in the cerebral hemispheres and less so posteriorally and inferiorally until it reaches its lowest level in the medulla oblongata. This conception is borne out by the observation of excised cerebral tissues which show a decreasing rate of oxygen intake as the neuraxis is descended<sup>12, 13</sup>. The oxygen consumption of various parts in the human brain *in vivo* will not be considered at this time because of conflicting results<sup>14, 15</sup>. Pending

the solution of this discrepancy we may point to another bit of evidence of a hierarchy in metabolic rate. In order to combat hypoglycemic coma carbohydrate must be administered and it has been observed that a larger amount of glucose is required to restore the functions of the cerebral hemispheres than for the subcortical areas<sup>16</sup>. Presumably a greater amount of foodstuff is necessary to support a higher rate of metabolism.

If we accept the concept of dissimilar metabolic rates it must follow that all parts of the brain will not be equally affected by hypoglycemia but that those regions with fastest rates would succumb first and those with the slowest, for example the medulla, last. Then in accord with Hughlings Jackson's idea¹ that the brain is so constructed that the higher anatomic and newer phyletic

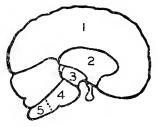


Fig. 1. Representation (transverse section) of the brain disclosing the five phyletic areas: 1. cerebral cortex; 2. subcorticodiencephalon; 3. midbrain; 4. pons and upper medulla; 5. medullary centers

portions contain areas which regulate and control the lower anatomic and older phyletic regions we might expect a series of release phenomena as each area in turn succumbs to an increasingly severe degree of carbohydrate deprivation<sup>17</sup>. Such a series is seen in the insulin hypoglycemia repeatedly produced in the pharmacologic treatment of schizophrenia<sup>18</sup>.

Following the injection of insulin the first phase involves the depression of the cerebral cortex (area I, Fig. I). Sensations become dull and abnormal, understanding is impaired and motor activity poor in execution. Contact with the environment is gradually lost as the patient becomes unconscious, the beginning of the second stage. The second group of signs proves to be due to a release of the functions in area 2, the subcorticodiencephalon. Three types of phenomena are observed in this stage. First are changes in motility reminiscent of those seen in a newborn baby with motor restlessness and primitive movements of many types such as involuntary sucking and involuntary grasping. Second there is increased sensitivity so that responses to stimuli become intense, excessive and at the same time lose direction. Finally, alterations in the autonomic system are seen with sympathetic predominance indicated by dilatation of the pupils, bulging of the eyeballs from their sockets, acceleration of the heart rate and rise of blood

pressure. This stage is not unlike that of sham rage exhibited by the decorticate animal. The third constellation (area 3) represents functions allocated to the midbrain. For example the body is seized by violent (tonic) spasms during which the legs become rigidly extended, the trunk is arched while the arms are thrust forward, bent at the elbows. The fourth group of manifestations, referable to the pons and upper portion of the medulla (area 4), begins when the arms are no longer held in front of the body but are slowly forced back over the head (extensor spasm). The back however is arched the legs are extended as in the third stage and the entire picture is similar to that of a decerebrate animal. Finally in the fifth stage (area 5) the cold, gray, clammy skin, the slow and feeble heart, the greatly depressed respiration, the muscular flaccidity, and the contracted pupils all give evidence that the metabolic depression is now affecting the vital medullary centers.

Soon after the fifth group of signs appear it is necessary to give the patient sugar. The blood glucose values rapidly rise and the brain once more obtains adequate supplies. The alterations in behaviour during recovery conform to the same plan as those seen during their development but this time their order is reversed.

It is well to make comparisons with the results of metabolic depression other than those produced by hypoglycemia. If the signs are due to a metabolic deficit then the same or at least a similar series of signs should be produced irrespective of the manner by which the metabolic deficit is created. As an example let us consider anoxia, a condition in which oxygen is no longer available to the brain in common with the other organs.

It is true that energy may be provided in the absence of oxygen, an anaerobic mechanism of great biological importance, for example, in sudden muscular activity. In the brain however, though not without significance<sup>19, 20, 21</sup>, the anaerobic release of energy is strictly limited for most of the energy usually available in the carbohydrate foodstuff of the brain, glucose, cannot be realized. For that reason the brain is highly sensitive to oxygen lack and when thus bereft of energy, can no longer support its own functions.

Whereas the signs of hypoglycemia may be observed over a period of 5 hours those of acute anoxia are more fleeting and must be limited to a period of as many minutes. Nevertheless the changes in behaviour follow the same general path of those of hypoglycemia and indicate a downward progression during anoxia and the reversed direction on recovery. These signs were demonstrated in a series of psychotic patients who respired undiluted nitrogen administered by means of a mask<sup>22</sup>. Early is seen a brief period during which consciousness becomes impaired as the cerebral hemispheres are the first to suffer from the decrease in available energy (area l, Fig. 1). The first phase ends as environmental contact is lost. With the loss of consciousness a series of dramatic neuromuscular reactions occurs beginning with a period of aimless motor restlessness which ensues after the subcorticodiencephalon acquires freedom from cortical restraint (area 2). Next come strong muscular contractions like those described in the third phase of hypoglycemic coma (tonic spasms) as the midbrain is freed from higher control (area 3). Finally emprosthotonos, flexion of the body, or opisthotonos, extreme extension, are seen in the fourth stage (area 4). These signs are release phenomena and indicate a decerebration of functional origin. At this point the inhalation of nitrogen is stopped to prevent involvement of the medullary centers. With the subsequent administration of air or oxygen the normal cerebral integrations are rapidly restored.

Supporting data for such a sequence of changes during hypoglycemia<sup>23</sup> or acute anoxia<sup>24</sup> is afforded by electroencephalographic tracings which reveal that the cortical rhythm vanishes before the subcortical. Conversely the administration of glucose or oxygen restores the subcortical waves before those of the cortex, additional evidence that the cerebral cortex workes at a higher rate of activity and has greater demands for energy than the subcortex.

Turning to the problem of pentothal anesthesia, we find that pentothal, like the

| Stage          | Anesthesia           | Characteristics                             | Site of<br>depression                   | Brain |
|----------------|----------------------|---------------------------------------------|-----------------------------------------|-------|
| I              | Clouding             | Euphoria loss<br>of<br>discrimination<br>to | Slight<br>depression<br>of cortex<br>to |       |
| 1              | Ciodang              | impairment of<br>environmental<br>contact   | moderate<br>depression<br>of cortex     |       |
| П              | Hyper<br>sensitivity | Loss of<br>consciousness                    | Predominant<br>control by<br>subcortex  |       |
| III<br>Plane I | Light surgical       | Hypoactivity to<br>painful stimulus         | Moderate<br>depressian<br>of subcortex  |       |
| PlaneⅡ         | Moderate<br>surgical | Loss of somatic<br>response to pain         | Predominant<br>control by<br>midbrain   |       |
| Plane∭         | Deep surgical        | Loss of visceral<br>response to pain        | Moderate<br>depression<br>of midbrain   |       |
| ъ              | Impending<br>failure | Fall in<br>pulse pressure                   | Moderate<br>depression<br>of pons       |       |

Fig. 2. A correlation between the stages of pentothal anesthesia and the outstanding clinical signs and their neuro-anatomic allocations

other barbiturates, exerts a metabolic inhibition which is most marked in the brain and relatively unimportant in other organs<sup>25</sup>. Measurements of brain metabolism made on human beings in the second and third stages of pentothal anesthesia disclose a decrease of approximately one-third<sup>15</sup>.

The barbiturates not only employ metabolic deprivation but also act on nerve function<sup>26</sup>. The latter action may be described as an elevation of the synaptic threshold<sup>27</sup> due perhaps to impeded recovery after impulse propagation<sup>28</sup>. Despite these diverse influences it is feasible to follow the events caused by metabolic depression.

In this brief exposition it is impossible to review the signs of pentothal anesthesia. Instead an explanatory diagram is inserted (Fig. 2). The figure is taken from a paper<sup>29</sup> in which it is suggested that the metabolic inhibition is the cause for certain similarities between barbiturate anesthesia and hypoglycemia or anoxia and especially so for the march of signs down the neuraxis with deepening anethesia. On the other hand the distinguishing characteristics of the anesthesia are attributed to the special effects which the barbiturate exert upon nerve functions.

Since the progression of the changes in behaviour observed following surgical or pharmacologic intervention seem to depend upon the hierarchy of metabolic rates in the various parts of the brain it is worth while to examine that phenomenon further. A clue as to its origin may be offered by a study of the changes in oxygen intake of the various parts of the brain during early growth. Animals which are born in an immature state, resembling man in that way, are appropriate material for a study of postnatal metabolic changes. The newborn rat, blind, poikilothermic and without righting reflexes, essentially a bulbospinal animal, can be followed through early growth while the later developed portions of the brain take on their due functions. The birth process marks the passage from intrauterine life to individual independence but does not necessarily represent a definite change in the fundamental patterns of growth and energy production.

Numerous in vitro studies of oxygen intake reveal a higher rate of metabolism in the adult than in the infant. This was first observed in infant rat brain<sup>30</sup>, and later confirmed on the dog<sup>13</sup>. These results indicate a rapid rise of cerebral metabolism in early life. The metabolic changes are the resultants of the distinctive rates in the discrete parts of the brain. It has been experimentally established that the metabolic rates are not equally affected by growth, but that each area possesses its own pattern of development. In experiments on the rat<sup>31</sup> and the dog<sup>13</sup> (Fig. 3) it was found that the lower parts of the brain are relatively more active than the higher ones at birth, and as development continues, the wave of metabolism presses forward so that the lower portions of the central nervous system are surpassed by the anatomically higher and phyletically more recently developed regions. The increasing rate of metabolism of the brain as a whole must therefore be attributed chiefly to the increasing rate in the newer parts of the brain during early life.

Additional evidence for this phyletic sequence can be observed by a study of the anaerobic metabolism. The short period of survival in anoxia observed in the mammal is made possible by the anaerobic production of energy which includes the splitting of carbohydrate to form lactic acid. The cerebral glycolytic rates are slowest in the newborn and increase to a maximum in early life<sup>32</sup>, <sup>33</sup>. In order to determine the contribution of each area in the brain making for this changing rate of glycolysis both dogs and cats were employed<sup>34</sup> and in several age groups: newborns to one week, three to seven weeks, three months, and adult. In general, the results of the experiments on dogs and cats

were similar. At birth the medulla oblongata revealed the highest glycolysis. In the adult, however, it is the cortex that shares the most rapid metabolic rate with the caudate nucleus.

The developmental progression observed in oxidation and glycolysis has also been found in the distribution of cerebral glycogen. Chemical determinations demonstrate that glycogen concentrations of the cerebral cortex and caudate nucleus increase with age. The percentage of glycogen in the lower parts, however, the cerebellum, medulla and spinal cord diminish progressively and are least in the adult<sup>35</sup>.

The quantitative analyses presented above show that both aerobic and anaerobic mechanisms are accelerated after birth. It seems probable that the more rapid rates are an expression of an increased concentration of enzymes. Such an increase can be

accounted for by the growing capacities of phosphorylase, phosphoglucomutase36, adenosine triphosphatase<sup>38</sup> and the cytochrome-cytochrome oxidase system37, 38 occurring in the brain during the early postnatal growth of the rat. Carbonic anhydrase though not found in the fetal rat is present in the adult where it is more plentiful in the functionally dominant cerebral areas than in the cord<sup>39</sup>. A study of fetal sheep proved that the enzyme cholinesterase is present in greater concentration in the spinal cord than the brain during early gestation. This relationship however is reversed in the last weeks before birth as the cholinesterase activities of the cord diminish while those of the brain far outstrip it40. This enzymatic evolution which appears earlier in the sheep than in the rat is not to be attributed solely to a difference in the enzyme studied in these two

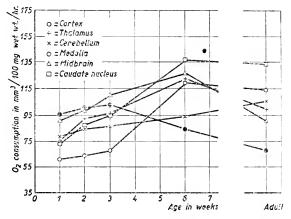


Fig. 3. Oxygen consumption vs. Age Dog Brain Parts. In the first week of life the highest rate of metabolism in the puppy's brain is found in the medulla; during the third week the midbrain assumes the highest oxygen consumption. From the fifth to the seventh week, the respiratory metabolism of all parts, with the exception of the medulla, is higher than the corresponding values for the first week of life and the caudate nucleus has advanced to the greatest oxygen intake up to this time. In the adult dog the latter still retains its prime position, while the cerebral cortex ascends to second place. The cerebellum, thalamus, midbrain and medulla follow in descending order.

species but it must also be remembered that the sheep is further advanced in the development of behavioural patterns at the time of birth.

To summarize, the increase in metabolic intensity does not occur in all parts of the brain simultaneously, but appears in the various portions at different times. The order of appearance is not a haphazard one but develops first in the posterior portions of the neuraxis and then progresses in an anterior direction. Such a stepwise passage advancing from the older to the newer parts of the brain recapitulates its phyletic development. Since many of the metabolic studies reviewed were made on newborns. It would seem that HAECKEL's dictum that ontogeny recapitulates phylogeny<sup>41</sup> should be broadened, in the case of the brain, and the time extended to include early postnatal growth with prenatal development.

To climb the phyletic ladder from our remotest ancestors through the fish, amphibia, reptiles and mammals, would entail a tremendous volume of description, which is not the point of this contribution. The general trend of this process of cephalization, or concentration of neural functions in the oral end of the animal, may be described briefly: as far back as the fish, brain is divided into five portions as it is in man, but in the fish and amphibia the chief site of integration for sensory and motor impulses lies in the midbrain. In these species the highest portion of the brain consists chiefly of the olfactory bulb, and the cerebral cortex which becomes all-important in man, is represented only by a thin layer of cells. On further ascending the phyletic scale to reptiles and birds as well as mammals, the subcortical structures immediately anterior to the midbrain become more prominent, as the organism achieves greater coordinating control. Lastly, the cerebral cortex, though getting off to a late start, gradually attains more complexity of structure and diversity of function until in the lower mammals it surpasses all other regions, and in the primates, especially in man, forms the largest and most complex part of the cerebral tissue. As this process of phylogeny is carried on from one species to another, no part of the neuraxis is scrapped, but each older part, in turn, comes under the influence of a later developed portion, which not only possesses finer discrimination and analysers but also plays a rôle in determining the motor expression of the older areas.

Though the brain of man as we see it today looks like a static structure, when it is examined more closely in the light of the phyletic conception, we see that it has come to its present construction as a result of a long series of accretions, beginning with the spinal cord and medulla oblongata and spreading in a cephalad direction, layer upon layer, until the cerebral hemispheres form the greatest mass of the brain. It is not to be supposed that each level is independent of its predecessors, but rather that it exists with a specific relation, both anatomically and physiologically, to the phyletically older portions<sup>42</sup>. Owing to this relation, the central nervous system may function as a unit, but a unity which is brought to a higher plane of integration with each successive step. The human brain is undoubtedly the latest arrangement of the central nervous system, but not necessarily the final one.

Sir Charles Sherrington<sup>43</sup> has expressed vividly Hughlings Jackson's conception. "That leading end, the head, has receiving stations signalling from things at a distance, things which the animal in its forward movement will next meet. A shell of its immediate future surrounds the animal's head. The nerve-nets in the head are therefore busy with signals from a shell of the outside world which the animal is about to enter and experience. The brain has thus arisen where signalling is busiest and is fraught most with the germ of futurity. Small wonder then that the brain plays a great rôle in the motor management of the muscle. Nerve management of muscle resolves itself largely into management of nerve by nerve, especially by brain, more and more so as evolution proceeds. With no greater equipment of muscle the superimposed amount of nerve becomes greater and greater; each new nerve-growth seems to entail further nerve-growth. Fresh organization roofs over prior organization. Brain is an example. 'So on our heels a fresh perfection treads'. But were it a government office we might be suspicious. This brain of ours is a perfect excrescence although our endowment of muscle remains but moderate".

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# THE DEVELOPMENT OF MUSCLE-CHEMISTRY, A LESSON IN NEUROPHYSIOLOGY

by

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In the development of muscle-chemistry four different periods can be distinguished: the pre-lactic acid era, the lactic acid era, the period of phosphorylations and the myosin period. The name of Otto Meyerhof is intimately connected with three of them. In no field of physiology has knowledge advanced so far towards the fundamental and elementary processes of function as in muscle chemistry. This advancement is mainly due to Otto Meyerhof's brilliant conception of chemical and physical aspects and to the unparalleled cooporation of two masterminds in different fields, Otto Meyerhof AND A. V. Hill.

In the prelactic acid era, although it starts paradoxically with Berzelius, who discovered in 1841 that muscles of exhausted deer contained more lactic acid than muscles of animals with partially paralysed extremities1, the rôle of lactic acid was quite unrecognized. There was even a very temperamental discussion as to what might be the fuel for muscular work. Fick and Wislicenus<sup>2</sup>, who climbed the Faulhorn (1956 m), between the lake of Brienz and the valley of Grindelwald, collected their urine and showed conclusively in a famous paper in 1865 that the excreted nitrogen corresponded only to 37 g of protein, which by no means accounted for the work done. This statement caused the long-held belief of Liebig, that protein is the source of muscular activity, to be discarded and attention to be drawn to carbohydrates. Six years later Weiss<sup>3</sup> showed that the glycogen content of muscle decreases with the work done, and it seems that Luchsinger<sup>4</sup> in Zürich was the first to recognize the importance of nutrition for the maintenance of a sufficient glycogen content of the muscles, and to point out that glycogen is the intermediate energy carrier between ingested foodstuffs and activity. The next step was only reached in 1803 when Panormoff<sup>5</sup> showed that glycogen in muscle is hydrolysed to glycose. Among the many original observations which Du Bois-REYMOND made, it seems that he was the first to recognize that a muscle becomes acid with activity and to relate this finding to Berzelius's observation of the formation of lactic acid<sup>6</sup>. It is quite amazing to see how, as early as 1850, a very clear conception existed and how it's development was delayed by the following accumulation of a great mass of very unimportant evidence up to the end of the century. This is even more surprising when we see that Heidenhain had found that the amount of lactic acid increased with the amount of work done. NASSE8 who seems to have had great influence at this time however believed that lactic acid was only formed in rigour and death, and did not recognize the importance of Helmholtz's fundamental finding that the alcoholic extract of muscle decreased with activity, whereas the aqueous extract increased, thus giving the first well founded evidence for chemical events, and suggesting that glucose and lactic acid increase at the expense of glycogen. It seems almost unbelievable that M. v. Frey wrote even in 1909 about chemical changes in muscular activity... "which acid is responsible cannot be stated to-day, since lactic acid seems not to account for it" (referring to the acidification of active muscle!)

The importance of phosphates seems to have been recognized for the first time by Salkowski<sup>10</sup>, who described the liberation of inorganic phosphate from an organic compound during activity, a finding which was rejected by v. Fürth, another of those most unfortunate cases (which occur so often!) where the authority of one man has delayed development.

It was MacLeod<sup>11</sup> who took up the point and found that inorganic phosphate increased and organic phosphate decreased, and Monari<sup>12</sup> first seems to have observed that the creatine-content of muscle increases with activity (phosphagen not being determined in his experiments). These—in our present point of view—most important findings could not be corroborated at that time to give a clear conception and were almost burried by a great deal of other chemical evidence which we consider to-day as entirely uninteresting and which filled the periodicals of the time.

The lactic acid era started in 1907 with the classical paper of Fletcher and HOPKINS<sup>13</sup>, in which they definitely established that fact that lactic acid is formed during activity and that it is absent (or practically absent) in resting muscles. This opened up a vast field and led to MEYERHOF's great work, which is summarized in a hypothesis, which was called the lactic acid theory of HILL AND MEYERHOF. The milestones of this development were the discoveries of the Pasteur-Meyerhof reaction, of the independence of initial heat of oxygen, the very accurate measurements of muscle heat by A. V. HILL and his colleagues, and their relation to chemical and calorimetric values obtained by MEYERHOF, the extensive study of lactic acid metabolism in muscle in all conditions of work, rigour and death, and finally the brilliant adaptation of this theory to muscular work in man by A. V. Hill<sup>14</sup> and his conception of oxygen-debt. It was a one-sided picture—as we all know to-day—and yet it is one of the golden pages of scientific discovery, because every new finding fitted into the theory and led to a very clear conception of what is taking place in a working muscle. It was very fortunate, that MEYERHOF published in 1930 his famous book on chemical events during muscle contraction, in which he gave an admirable account of the lactic-acid hypothesis<sup>15</sup>.

The year 1930 brought, what A. V. Hill called the revolution in muscle physiology. Lundsgaard's 16 paper on mono-iodoacetic acid poisoned muscles and the absence of lactic acid formation in these muscles was—as it seemed at first—a heavy blow to the lactic acid hypothesis. It is very interesting to read to-day the conclusions Bethe 17 drew at that time and it is equally astonishing to see, how quickly Meyerhof reacted and how he and Lundsgaard kept the lead. The conception of energetic coupling between different reactions was worked out and proved to be a new and extremely useful aspect in the classification and understanding of the chemical events including adenylpyrophosphate, creatinghosphate and fructosediphosphate breakdown. Ritchie 18 introduced the idea that all chemical events might be recovery processes and therefore furnish the energy for the next contraction. This led to the conception that energetically coupled reactions furnish in steps the necessary free energy to restore the energyloss which occurs in an explosive way during contraction. This conception has been recently summarized by Meyerhor 19 in an article which contains all the classical points of view

of the era. This era might be called the period of phosphorylations and it is characterized by the discovery of the Parnas-reaction, the Lohmann-reaction and the complete series of steps in glycolysis in muscle, with the isolation of the corresponding enzymes.

In 1939 the myosin period started with the paper of Engelhardt and Ljubimova<sup>20</sup>, which was followed by Szent-Györgyi and Banga's<sup>21</sup>, Needham's<sup>22</sup>, Bailey's<sup>23</sup> and Kleinzeller's<sup>24</sup> papers. Myosin, the "muscle machine" or what A. V. Hill has always called the fundamental process, became the center of attention. Myosin had been known, of course, for quite a long time. In 1930 my friend John Edsall and I published experiments, which showed that myosin must be the contractile element of muscle. The important point about Engelhardt and Ljubimova's paper is, however, that they found that the enzyme associated with the breakdown of ATP was associated with myosin. With this it became evident at once that there is a close relation between the "muscle machine" and the whole set of coupled chemical reactions. Szent-Györgyi and his coworkers<sup>25</sup> have added a great deal of very interesting new information about the nature of the muscle machine and thus we are just now in the midst of a "myosin era". Meyerhof has attached his name to this period by the almost simultaneous isolation of ATP-ase from myosin, first described by Price and Cori<sup>26</sup>.

What is the lesson neurophysiology can learn from this development?

- I. A rather long period of widespread chemical research has to precede the definite identification of those chemical reactions which are really essential. I am afraid that the smallness of nerve and the impossibility to accumulate break-down products connected with the absence of fatigue in peripheral nerve has prevented any extensive chemical work. Such work preceded the lactic acid era in muscle chemistry. The ground for neurophysiology therefore is not as well prepared as it was for muscle-physiology in 1907.
- 2. Once the importance of lactic acid was established, an intensive attack was made from all sides, yielding an astounding amount of information. Looking back it can well be said, that the prejudiced concentration on lactic acid was very much worthwhile! Is acetylcholine in neurophysiology a problem which will prove to be as fruitful as lactic acid was in muscle physiology? I doubt it and I realize that in this respect I disagree with my colleague Nachmansohn<sup>27</sup> who has published an admirable amount of work on the subject.
- 3. In muscle the energy expenditure is the main function. In nerve, nature gives us an opposite example of maximal economy in energy expenditure connected with function. The energy changes are so small that it took even A. V. HILL 15 years to measure them. This renders the task of corroboration between physical and chemical events in nervous excitation extremely difficult and tedious.
- 4. In muscle physiology it was possible to study the interesting reactions in vitro, to measure the various steps of glycolysis and to isolate the important enzyme-systems. Sodium fluoride and isoacetic acid have been powerful tools in this work. In nervephysiology the material is complex and there is, as far as I can see, no definite clue to any chemical reaction of primary importance. Gerard<sup>28</sup> has contributed most valuable studies on nerve-chemistry by working along lines similar to those used by muscle physiologists, but I think he will agree with me in saying, that our knowledge of what is going on chemically in order to restore the energy expenditure of the ionic changes (potassium going "out", sodium going "in" and vice versa, cf. Hodgkin<sup>29</sup>) is very far from being satisfactory. I think it is well to emphasize that brain-brei is in no way a

model for peripheral nerve chemistry and that the application of results obtained with brain-brei must be regarded with caution.

5. Physical phenomena, accompanying the chemical changes have been a great help in establishing the sequence of events in muscle. Volume change, change of p<sub>H</sub>, variation of birefringence, of light scattering and change of electrical resistance have been studied with great success, and it is one of the outstanding characteristics of MEYERHOF's work that he always was able to make a fruitful correlation between these phenomena and the chemical aspect. In nerve, all these effects—if they exist at all—are probably extremely small. David Hill (personal communication) has been able to detect changes of light scattering and volume changes in certain nerves. This may be the beginning of a new development. But on the whole,—except for action potentials—the nerve does not offer many good points for attack from the physical side.

The problem of the function of nerve remains, as A. V. Hill<sup>30</sup> has stated 17 years ago, intellectually quite a respectable one. For all those who are attracted by it the study of the development of muscle chemistry is a lesson of how to proceed. Otto Meyerhof's lifework with its unique combination of physical and chemical aspects furnishes the pattern which must be followed, if we want to understand what "excitation" really means.

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### PART III

# DRUG ACTION

# SUBSTRATE SPECIFICITY OF AMINO-ACID DECARBOXYLASES

by

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During the last two years a number of observations on substrates of amino-acid decarboxylases have been recorded from this laboratory. In this review the attempt is made to correlate the results obtained and to arrive at conclusions of a more general character. The experimental data and the methods used have been described elsewhere (Blaschko, Holton, and Sloane Stanley<sup>1, 2</sup>; Blaschko<sup>3</sup>; Sloane Stanley<sup>4, 5</sup>).

The decarboxylation of L-3:4-dihydroxyphenylalanine (DOPA) is catalysed by two enzymes: the mammalian L-DOPA-decarboxylase (Holtz, Heise, and Lüdtke<sup>6</sup>) and the bacterial L-tyrosine decarboxylase (Epps<sup>7</sup>). The two enzymes differ in their affinity for L-tyrosine: this is probably the "natural" substrate of the bacterial enzyme, but it is not attacked by the mammalian enzyme. The difference in substrate specificity of the two enzymes has been studied more systematically.

The experimental procedure adopted is easily described. As a source of the bacterial enzyme we used an acetone-dried preparation of *Streptococcus fæcalis* R (ATCC 4083); we owe this strain to Professor I. C. Gunsalus. The bacteria were usually grown in a medium free of vitamin  $B_6$ ; in these preparations the tyrosine apodecarboxylase was present, but had to be completed by the addition *in vitro* of pyridoxal and ATP. In some of the experiments we used a "complete" preparation obtained from cells grown in the presence of pyridoxal. As a source of the mammalian DOPA decarboxylase we used fresh tissue extracts, from guinea-pigs kidney or from rats liver.

The enzymic decarboxylation of each amino-acid was measured by following the time course of CO<sub>2</sub> formation manometrically. If an amino-acid was found to be decarboxylated, the contents of the manometer flasks were used for a determination of the pharmacological activity of the amine formed. The activity was tested on the arterial blood pressure of the spinal cat; the pressor activity of the amine formed by enzyme action was compared with that of the synthetic amine.

# I. MONOHYDROXYPHENYLALANINES

Our results are summarized on Table I. It was found that *m*-hydroxyphenylalanine (the "meta-tyrosine" of Blum<sup>8</sup>) was a substrate of the mammalian enzyme; the rate of decarboxylation was slightly less than with 3:4-dihydroxyphenylalanine as substrate. The bacterial preparation also acted on *m*-hydroxyphenylalanine, at about one-third of the rate of decarboxylation of tyrosine.

In the mammalian tissue extracts, o-hydroxyphenylalanine (Blum's $^8$  "orthotyrosine") was decarboxylated at approximately the same rate as the meta hydroxy

derivative. With the bacterial preparations, the rate of  $CO_2$  formation from o-hydroxy-phenylalanine was practically zero.

TABLE I

DECARBOXYLATION OF TYROSINE AND ITS ISOMERS

+ signifies decarboxylation

signifies no decarboxylation

|                       | Substrates                                 |                                                                                                         |                                              |  |  |  |
|-----------------------|--------------------------------------------|---------------------------------------------------------------------------------------------------------|----------------------------------------------|--|--|--|
|                       | OH  CH <sub>2</sub> CHNH <sub>2</sub> COOH | $\begin{array}{c} \text{OH} \\ \text{CH}_2 \\ \mid \\ \text{CHNH}_2 \\ \mid \\ \text{COOH} \end{array}$ | CH <sub>2</sub><br>CHNH <sub>2</sub><br>COOH |  |  |  |
| Bacterial preparation | -                                          |                                                                                                         |                                              |  |  |  |
| Mammalian preparation |                                            | <del>-</del>                                                                                            |                                              |  |  |  |

Results of competition experiments suggest that the two enzymes responsible for these decarboxylation reactions are the bacterial tyrosine decarboxylase and the mammalian DOPA decarboxylase. One molecule of each DL-amino-acid gives one-half of a molecule of CO<sub>2</sub> formed; we therefore assume that only one of the two steroisomers, the L-form, is decarboxylated.

These findings demonstrate the importance of the phenolic hydroxyl groups and their positions on the benzene ring for the reaction between enzyme and substrate. It seems safe to assume that these groups react with the protein part of the decarboxylase system.

The nature of the forces which are at work between enzyme protein and substrate is not known. In the case under consideration, it seems possible that the reaction between the phenolic hydroxyl groups and the enzyme involves the formation of a hydrogen bond, with the hydroxyl group either as a "donor" or an "acceptor". At any rate, the results obtained can be understood if it is assumed that the substrate must be held by a group in the enzyme situated so that it can react with a hydroxyl group in

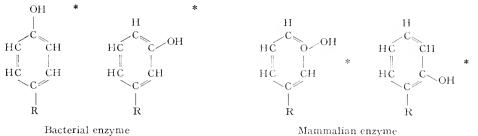


Fig. 1. The asterisk marks the position of the active group in the enzyme in relation to the substrate. References p. 136/137.

one of two adjacent positions on the benzene ring. The position of this group in the enzyme would be different for the bacterial and the mammalian enzyme, as shown in Fig. 1.

#### II. 2:5-DIHYDROXYPHENYLALANINE

This amino-acid has recently been synthetized by Neuberger. We have examined it and have found that it is a substrate of the mammalian enzyme, but that it is not a substrate of the bacterial enzyme.

That 2:5-dihydroxyphenylalanine is a substrate of the mammalian decarboxylase is easily explained by the hypothesis outlined above; the lack of affinity for the bacterial enzyme, however, is not obvious; possibly the presence of the hydroxyl group in ortho position interferes with the attachment to the enzyme.

We have examined both the L and the D forms of this amino-acid; in agreement with expectation, only the L form is a substrate of DOPA decarboxylase. The product of the decarboxylation reaction,  $\beta$ -2:5-dihydroxyphenylethylamine, seems to be a substrate of amine oxidase; this suggests that in the living animal it is metabolized as follows:

HO

$$CH_2$$
-CHNH $_2$ -COOH

 $CH_2$ -CHNH $_2$ -COOH

 $CH_2$ -CH $_2$ -NH $_2$ 
 $OH$ 
 $\beta$ -2:5-dihydroxyphenylethylamine

 $OH$ 
 $O$ 

It has been shown that the amino-acid gives rise to homogentisic acid in the alcaptonuric subject (Neuberger, Rimington, and Wilson<sup>10</sup>). In normal animals and human subjects, both the amino-acid and the corresponding amine are fully metabolized (Neuberger<sup>9</sup>; Leaf and Neuberger<sup>11</sup>). This aspect of our findings has been more fully discussed elsewhere (Blaschko *et al.*<sup>2</sup>).

#### III. 3:4-DIHYDROXYPHENYLSERINE (NORADRENALINE CARBOXYLIC ACID)

The study of this compound has revealed another difference between the mammalian and the bacterial decarboxylase<sup>1</sup>. On decarboxylation, it yields noradrenaline:

It was found that the amino-acid was not decarboxylated by extracts of mammalian tissues; it was, however, decarboxylated by the bacterial preparation. The rate of CO<sub>2</sub> formation with dihydroxyphenylserine was much slower than with tyrosine as substrate, but the decarboxylation was almost quantitative; approximately one-half of the racemic substance was decarboxylated. The biological assay on the arterial blood pressure of the spinal cat, together with the measurement of the amount of CO<sub>2</sub> formed, showed that the amine formed was laevo-noradrenaline.

#### IV. N-METHYLATED AMINO-ACIDS

Ten years ago, the observation was made that the introduction of a N-methyl group abolished the substrate specifity for DOPA decarboxylase (Blaschko<sup>12</sup>). Preparations of mammalian liver and kidney which had DOPA decarboxylase activity were found not to act on N-methyl-3:4-dihydroxyphenylalanine:

$$HO$$
 CHOH-CHNH(CH3)-COOH

This observation was made the basis of a scheme of biosynthesis of sympathin and adrenaline. It had often been assumed previously that the formation of adrenaline involved a decarboxylation reaction, but it was now shown that the body was not able to produce a secondary amine by direct decarboxylation of the N-methyl-amino-acid, whereas it was able to produce the corresponding primary amine. Primary amines with sympathicomimetic activity were therefore postulated as intermediary products in adrenaline synthesis. Earlier already, pharmacologists had discussed the possibility of the identity of Cannon's "sympathin E" with noradrenaline (Bacq¹³; Stehle and Ellsworth¹⁴). The biochemical findings gave a simple explanation for the occurrence of this substance.

Two amino-acids were studied in 1939: N-methyl-dihydroxyphenylalanine and N-methyl-tyrosine. One important methylamino-acid, however, was not available at that time; this was N-methyl-3:4-dihydroxyphenylserine. Already in 1906, FRIEDMANN<sup>15</sup> had considered this acid as a possible precursor of adrenaline; he suggested that adrenaline was formed in the reaction:

This suggestion could not be tested by experiment until the synthesis of adrenaline References p. 136/137.

carboxylic acid was achieved by Dalgliesh and Mann<sup>16</sup>. We have recently examined this compound. It was found not to be decarboxylated by a number of mammalian tissue extracts and, unlike the corresponding amino-acid, dihydroxyphenylserine, it was not a substrate of the bacterial enzyme preparation.

The substrate specificity of DOPA decarboxylase in connexion with pathways of adrenaline synthesis has recently been reviewed elsewhere (Blaschko<sup>17</sup>). Two possible ultimate precursors of adrenaline were discussed: noradrenaline and N-methyl-3:4-dihydroxyphenylethylamine (also known as epinine):

The rôle of epinine in the biosynthesis of adrenaline has recently been discussed by Danneel<sup>18</sup> and by Holtz and Kroneberg<sup>19</sup>. The presence of this substance in mammalian tissue has never been demonstrated. Recently, noradrenaline has been found in human tumours of the suprarenal medulla (Holton<sup>20</sup>) as well as in the suprarenal gland (Schümann<sup>21</sup>). Evidence is also accumulating that both adrenaline and noradrenaline are released from the suprarenal medulla (Meier and Bein<sup>22</sup>; Bülbring. and Burn<sup>23</sup>; Holtz and Schümann<sup>24</sup>).

#### V. DOPA DECARBOXYLASE AND PYRIDOXINE DEFICIENCY

Like the mammalian enzyme, the bacterial enzyme does not act on N-methyltyrosine (Epps<sup>7</sup>) and N-methyl-dihydroxyphenylserine. This suggests that the inability to act on N-methyl-amino-acids is due to a property common to both enzymes.

It is known that the bacterial codecarboxylase (Gale and Epps<sup>25</sup>), the prosthetic group of the bacterial tyrosine decarboxylase, is pyridoxal phosphate (Gunsalus, Bellamy and Umbreit<sup>26</sup>). Green, Leloir, and Nocito<sup>27</sup> achieved a partial purification of DOPA decarboxylase and a reactivation of the apoenzyme by pyridoxal phosphate. It is, however, not generally accepted that DOPA decarboxylase contains pyridoxal phosphate (see Martin and Beiler<sup>28</sup>; Work and Work<sup>20</sup>).

When the DOPA decarboxylase activity was determined in liver extracts of rats reared on a diet deficient in pyridoxine (vitamine B<sub>6</sub>), enzymic activity was found to be low, and in a few of the extracts the activity had practically disappeared (Blaschko, Carter, O'Brien, and Sloane Stanley<sup>30</sup>; and unpublished observations). Addition of pyridoxal plus ATP in vitro brought about a partial restoration of the enzymic activity. More recently, through the kindness of Dr K. Folkers, we have been able to test the effect of synthetic codecarboxylase: we have found that it is possible to restore the activity of the extracts from B<sub>6</sub>-deficient animals to normal values by the addition in vitro of 10  $\mu$ g of synthetic codecarboxylase to the equivalent of 550 mg of fresh weight of liver. These experiments allow us to conclude that DOPA decarboxylase, like the bacterial tyrosine decarboxylase, contains pyridoxal phosphate.

There is experimental support for a suggestion by SNELL<sup>31</sup> that in transamination the initial reaction between amino-acid and pyridoxal phosphate involves the formation of a  $-N = C\langle$  bond. In analogy, it seems likely that the decarboxylation requires a reaction between the amino group of the amino-acid and the aldehyde group of pyridoxal phosphate:

It is clear that this reaction will only occur when the amino group is unsubstituted. We conclude that N-methyl-amino-acids are unable to react with the formation of a  $-N = C\langle$  bond. This inability would account for the fact that N-methyl-amino-acids are not substrates of the amino-acid decarboxylases.

#### VI. THE BASIS OF SUBSTRATE SPECIFICITY

The experiments discussed have shown two different types of substrate specificity. DOPA decarboxylase may serve to demonstrate these:

- a. tyrosine is not a substrate of DOPA decarboxylase, because it does not react with the enzyme protein;
- b. N-methyl-3:4-dihydroxyphenylalanine is not a substrate of DOPA decarboxylase, because it does not react with the coenzyme.

DOPA decarboxylase, like all the amino-acid decarboxylases, presents a *third* type of substrate specificity: specificity for the members of the L series. Holtz, Heise, and Lüdtke<sup>6</sup> suggested already that DOPA decarboxylase was specific for L-dihydroxyphenylalanine; we have confirmed this, using the D isomer which was not decarboxylated (Blaschko<sup>32</sup>).

The lack of affinity for the D form is easily understood in the light of the evidence discussed in this review. If we consider the alpha carbon atom of the amino-acid,

$$\begin{array}{c} \mathbf{R} \\ |_{a} \\ \mathbf{H} - \mathbf{C} - \mathbf{N} \mathbf{H_{2}} \\ | \\ \mathbf{COOH} \end{array}$$

we see that three of the groups attached to this atom take part in the decarboxylation reaction:

- a. the carboxy group, which loses carbon dioxide,
- b. the amino group which reacts with the aldehyde group of pyridoxal, and
- c. the group R which reacts with the enzyme protein.

If the decarboxylation requires a fixed relationship of these three groups relative to the enzyme, it is clear that the L and D forms are not equivalent; only one of the stereoisomers can be expected to fulfil the conditions required for decarboxylation. The stereospecificity of other enzymes dealing with amino-acids may have a similar basis (see Rydox<sup>33</sup>), but the conditions of specificity are not so completely known.

It has been pointed out that the presence of a third polar group in R is a common feature of all bacterial amino-acid decarboxylases (Gale<sup>34</sup>). The same is true for the mammalian decarboxylases, not only for DOPA decarboxylase, but also for the L-cysteic decarboxylase of mammalian liver (Blaschko<sup>35</sup>).

References p. 136/137.

#### Acknowledgement

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#### SUMMARY

- 1. The decarboxylation by bacterial and mammalian enzymes of a number of amino-acids structurally related to tyrosine has been studied.
- 2. The position of the phenolic hydroxyl group in tyrosine and its isomers is shown to determine substrate specificity. This is explained by a reaction between the OH group of the substrate and the enzyme protein.
- 3. Methylamino-acids are not decarboxylated; this is explained by their inability to react with the aldehyde group in pyridoxal phosphate (codecarboxylase).
- 4. The stereospecificity of the amino-acid decarboxylases is discussed on the basis of these observations.

#### RÉSUMÉ

- 1. La décarboxylation de quelques acides aminés, apparentés à la tyrosine, a été étudiée au moyen de ferments bactériens et animaux.
- 2. La position des groupes OH dans la tyrosine et ses isomères est déterminante pour la spécificité des décarboxylases. Nous en déduisons que la réaction entre l'apoferment et les acides aminés en question a lieu au niveau du groupe OH.
- 3. Les acides méthyl-aminés ne sont pas décarboxylés en présence de ces ferments. Ce phénomène s'explique par l'impossibilité du groupe N-méthylique de réagir avec l'aldéhyde du phosphate de pyridoxal (codécarboxylase).
- 4. Les résultats de ce travail nous permettent de discuter le phénomène de la stéréospécificité des décarboxylases.

#### ZUSAMMENFASSUNG

- 1. Die Decarboxylierung einiger dem Tyrosin verwandter Aminosäuren durch tierische und bakterielle Fermente wurde untersucht.
- 2. Die Position der phenolischen Hydroxylgruppe des Tyrosins und seiner Isomeren ist für die Substratspezifität von Bedeutung. Diese Beobachtung wird erklärt durch die Annahme einer Bindung zwischen der OH-Gruppe des Substrats und dem Apoferment.
- 3. Methylaminosäuren werden nicht decarboxyliert; dies wird erklärt durch das Ausbleiben der Reaktion mit der Aldehydgruppe des Pyridoxal-Phosphats ("Codecarboxylase").
- 4. Die Stereospezifität der Aminosäuredecarboxylasen wird im Lichte der gewonnenen Resultate erläutert.

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#### GLYCOLYSIS IN PHARMACOLOGY1, 2

by

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Classical pharmacology deals with the action of drugs on organ systems. If the question is raised as to why a certain drug acts on a particular organ system, the answer may only be obtained by searching for some system inside the cell which is sensitive to the drug in question. The most fruitful line of endeavor has been to test the affect of the drug on enzyme systems known to be involved in cellular metabolism. Many pharmacological actions of drugs can be explained in this manner. For example, the pharmacological properties of vitamins, physostigmine, BAL, and cyanide have been explained to everyone's satisfaction on an enzymatic basis. During the past war, a great deal of attention was paid to the action of antimalarial drugs, ionizing radiation and chemical warfare agents on enzymatic processes. In fact, there is a growing school in Pharmacology which has for its main purpose the localization of drug action on enzymatic processes. Some of this work has been reviewed by GREEN¹, BERNHEIM², CLARK³, and McElroy⁴. The recent book by Work and Work⁵ is an excellent example of the development of this field in chemotherapy.

Welch and Bueding<sup>6</sup> have laid down very severe criteria which should be met before the action of a drug can be attributed to its effects on an enzyme system. These criteria involve concentrations, organ and tissue specificity and close parallelism between the activity of structurally related compounds. These criteria are very hard to meet in this field. It is very difficult to determine how much drug is acting on a specific organ when the drug is administered to the whole animal. When working on enzyme systems, cell interfaces are destroyed and permeability is no longer a question, which may modify drug action. Therefore, the criteria of Welch and Bueding<sup>6</sup> should be used as an ultimate goal and not be used to delay or to give up work and thinking in this field.

It is the purpose of this article to give several examples of drug action on the glycolytic system in order to show how the discoveries of Meyerhof are now being used in Pharmacology. Meyerhof used many pharmacological agents as chemical tools in his work on muscle metabolism. Narcotics, methylene blue, chloroform, caffeine, and moniodoacetic acid are a few of many agents employed in his work. More recently Meyerhof and his associates have employed alloxan<sup>8</sup> in their study of glycolysis of brain preparations and have reported<sup>9</sup> the effects of potassium 1, 2-naphthoquinone-4-sulfonate on the respiration and glycolysis of *Trypanosoma equiperdum*.

<sup>&</sup>lt;sup>1</sup> Read before a Seminar at the Army Chemical Center, March 9, 1949.

<sup>&</sup>lt;sup>2</sup> In this paper, the term "glycolysis" is used in the general meaning for the break down of any carbohydrate into lactic acid by enzymatic processes.

Any abnormal cell, invading organism or abnormal metabolic event in the body involving or using carbohydrate opens itself to this mode of attack, namely, to find a chemical substance which will block or modify its use of carbohydrate but not affect the use of carbohydrate by the normal cells of the host. In this manner the abnormal cells or invading organisms can no longer use sugar for energy purposes and thus are destroyed. Abnormal metabolism of carbohydrate may also be checked or diverted into normal pathways in a similar manner. Since the carbohydrate is generally oxidized by the invading organisms, two possibilities are available for blocking by enzymatic inhibitors; a) in the oxidative chain and b) in the glycolytic system. In the cancer field, for example, if an agent could be found which will block the use of glucose either by oxidation or by glycolysis in the rapidly growing cells, growth would cease since these cells depend mainly on the metabolism of glucose for their growth. Therefore, there should be a constant search for compounds which inhibit glycolysis or the oxidation of various sugars. Such a search may some day be rewarded with a differential inhibitor which will block sugar utilization in the cancerous cell and not in the normal cell. Such inhibitors have been found already for certain invading organisms and may well be found for the cancer cell. A review of some of the literature in this field up to 1938 has been made by Gemmill<sup>10</sup>.

Quinine and Atabrine: During the war, Evans and his associates made a very intensive study of quinine and atabrine on glycolysis. This group demonstrated that the glycolysis of the malarial parasite was similar to that of the phosphorylating glycolysis of yeast and muscle<sup>11</sup>. Following these observations the effects of quinine and atabrine were investigated12 on this system from malarial parasites, yeast and mammalian muscle. Atabrine inhibited hexokinase activity and the lactate dehydrogenase in the parasite preparations. Both quinine and atabrine inhibited the yeast hexokinase while quinine was inhibitory to the phosphorylase and the phosphoglucomutase from rabbit's muscle. Lactate dehydrogenase from beef heart was very susceptible to atabrine action. However, from the concentrations needed to inhibit these enzymes in the glycolytic systems, these authors concluded that the therapeutic site of inhibition is probably in the oxidative cycle unless there is a possibility of a high concentration of these drugs localizing inside the parasite cell. Bovarnick, Lindsay, and Hellerman<sup>13</sup> attribute the inhibitory action of atabrine on the oxidation of glucose to an interference of phosphorylation which is essential before glucose may be oxidized by the malarial parasite.

Naphthoquinones: There has been considerable attention given to the naphthoquinones in pharmacology in recent years. In addition to the discovery that vitamin K has a naphthoquinone nucleus, these compounds have been investigated for their antimalarial<sup>14</sup>, fungicidal<sup>15</sup>, antitubercular<sup>16</sup>, and antibacterial actions<sup>17</sup>. Some of the naphthoquinones have the power to inhibit mitosis which makes them of interest from the standpoint of tumor growth<sup>18</sup>. Naphthoquinones inhibit acid formation in the saliva which may aid in the prevention of tooth decay<sup>19</sup>.

Considerable work has been done to explain the action of naphthoquinones on a possible enzymatic site. Wendel<sup>20</sup> has described an inhibition of the oxygen uptake and the use of carbohydrate in red blood cells parasitized with a malarial parasite. Ball, Anfinsen, and Cooper<sup>21</sup> have made an extensive study of the inhibition of oxygen uptake and have come to the conclusion that the inhibitory site is between cytochrome c and b in the chain of respiratory enzymes. Bueding, Peters, and Waite<sup>22</sup>

have shown that 2-methyl-1,4-naphthoquinone inhibits aerobic glycolysis in *Schistosoma mansoni*, in vitro. Warren<sup>23</sup> has observed a similar effect in bone marrow. Meyerhof and Randall<sup>9</sup> have found an inhibition of respiration, glycolysis and motility of *Trypanosoma cquiperdum*, in vitro, using potassium 1,2-naphthoquinone-4-sulfonate. Gemmill<sup>24</sup> has studied the effects of various naphthoquinones on anerobic glycolysis of frog muscle. His results are given in Table 1.

#### TABLE 1

Naphthoguinones which inhibited glycolysis in concentrations of  $1\cdot 10^{-3}$  molar or less

- 1. Sodium 1,2-naphthoquinone-4-sulfonate
- 2. 2-methyl-1,4-naphthoquinone
- 3. Sodium 2-methyl-1,4-naphthohydroquinone diphosphate
- 4. 2-hydroxy-3-methyl-1,4-naphthoquinone (Phthiocol)
- 5. 2-methyl-4-amino-1-naphthol hydrochloride
- 6. 2-hydroxy-1,4-naphthoquinone (Lawsone)
- 7. 1,4-naphthohydroquinone
- 8. 2-methyl-3-bromo-1,4-naphthoquinone
- 9. 2-chloro-3-N-thiobutyl-1,4-naphthoquinone
- 10. 2-methyl-3-thioethyl-1,4-naphthoquinone
- 11. 2-hydroxy-3-cyclohexanol-1,4-naphthoquinone

In Table I may be seen several napthoquinones which are glycolytic inhibitors. The relationship of concentration to inhibition by sodium 1,2-naphthoquinone-4-sulfonate may be seen in Fig. 1. At low concentrations there is a slight stimulation of glycolysis. As the concentrations increase there is a marked change in glycolysis with practically complete inhibition occurring with concentration of  $0.4 \cdot 10^{-3}$  Molar. Some

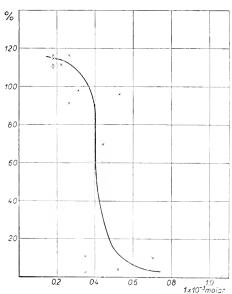


Fig. 1. The effects of increasing concentrations of sodium 1,2-naphthoquinone-4-sulfonate on glycolysis. Abscissae, 1·10<sup>-3</sup> Molar final concentration: ordinates, per cent of normal glycolysis.

of the naphthoquinones which have vitamin K activity also are inhibitors of anerobic glycolysis: 2-methyl-1,4-napthoquinone, sodium 2-methyl-1,4-naphthoquinone diphosphate and 2-methyl-4-amino-1-naphthol hydrochloride. Another interesting fact which came out of this work was that the attachment of a halogen in the 2 or 3 position increased the inhibitory activity of these compounds.

Amidines and Related Compounds: Historically, the study of the chemotherapeutic properties of the diamidine compounds was a direct result of a search for agents which would block the use of glucose by the trypanosomes<sup>25</sup>. The early discovery that decamethylene diguanidine hydrochloride (Synthalin) was effective against certain trypanosomes led to a search for less toxic substances. Out of this search came many guanidines, isothioureas, amidines<sup>26</sup> and numerous aromatic diamidines, among them being stilbamidine and pentamidine. It was soon

shown that doses of the diamidines which were active against trypanosomes did not produce a fall in blood sugar of the host. Therefore, attention was given to the sugar metabolism and oxygen utilization of these organisms. Lourie and Yorke<sup>27</sup> have stated that the diamidines may block the aerobic glucose metabolism in the diamidinesensitive species. The diamidine-insensitive species would be capable of obtaining their energy from the anerobic glycolysis in the presence of the drug.

Some attention has been paid to the possible enzymatic site of the action of these compounds. Blaschko and Duthie<sup>28</sup> have found an inhibitory action of the various amidine derivatives on the amine oxidase activity of the rabbits' liver. Bernheim<sup>29</sup> has shown that the oxidation of proline and alanine by *E. coli* is inhibited by propamidine. However, the oxidation of glucose, pyruvate and succinate is not affected by this drug. Dickens<sup>30</sup> has demonstrated that guanidine carbonate increases the aerobic glycolysis of the rat brain cortex. These facts led to a study of the effects of diamidines and related compounds on anerobic glycolysis of glycogen to lactate in muscle extract (Gemmill<sup>31</sup>). The various compounds in this series which inhibited glycolysis are given in Table II. In the same paper is given a list of styryl and cyanine compounds which are active inhibitors.

| Diamidines:             | Diguanidines:       |
|-------------------------|---------------------|
| C <sub>12</sub> .2 HCl  | Diguanidine HC      |
| C <sub>13</sub> .2HCl   | C <sub>12</sub> HCl |
| Monoguanidines:         | Diisothioureas:     |
| Guanidine HCl           | $C_{10} HBr$        |
| Methylguanidine sulfate | C <sub>12</sub> HBr |
| Arginine HCl            | Stilbamidine        |
| C <sub>8</sub> HCl      | Pentamidine         |
| C <sub>13</sub> HCl     | Chlorguanidine      |

Alloxan: Since the discovery that alloxan may produce diabetes by destroying the cells in the islets of Langerhans, there has been a renewed interest in the effect of alloxan on enzyme systems. Purr³² has demonstrated that alloxan has the ability to inhibit papain and cathepsin and Hopkins, Morgan, and Lutwak-Mann³³ have shown the same effect on the succinic dehydrogenase. Alloxan may act as a hydrogen acceptor in enzyme solutions³⁴, ³⁵. Gemmill³⁶ has demonstrated that alloxan may inhibit glycolysis. The degree of inhibition was proportional to the concentration of alloxan and the inhibition was partially reversed by cysteine. Therefore alloxan may be added to the group of oxidizing agents which can reversibly inactivate glycolysis. It would be of interest to show that the cells in the islets of Langerhans have a glycolytic system which was very sensitive to this reagent.

Caffeine: Considerable work has been done on the effect of caffeine on glycolysis in the intact muscle. Meyerhof<sup>37</sup> demonstrated that caffeine increased lactate formation. Matsuoka<sup>38</sup> continued and reported in detail this demonstration. David<sup>39</sup> has shown a large increase in lactate formation in caffeine contracture. Gemmill<sup>40</sup>, in cell free extracts, was able to demonstrate that caffeine and some theobromine derivatives caused an increase in the rate of glycolysis which was followed by an inhibition.

Mercury Compounds: GEMMILL AND HELLERMAN<sup>41</sup> studied the effects of small concentrations of phenylmercuric hydroxide, p-chloromercuric benzoic acid and mercuric chloride on glycolysis in muscle extracts. These substances have the power to inhibit glycolysis and the inhibition is abolished by the addition of cysteine.

Iodine: In the same paper in which the action of the mercury compounds on glycolysis was described, Gemmill and Hellerman<sup>41</sup> also demonstrated that small amounts of iodine inhibited glycolysis. This effect was reversed by the addition of cysteine. Lipmann<sup>42</sup> had previously shown that iodine was an active inhibitor of glycolysis. Rapkine<sup>43</sup> traced the action of oxidizing agents to the oxidoreduction between phosphoglyceraldehyde and pyruvic acid. Lipmann<sup>44</sup> has pointed out that there are five enzymes in the glycolytic system which may undergo oxidative inactivation and reactivation with glutathione.

Anesthetics: Watts<sup>45</sup>, working in this laboratory, has shown that methadon and nupercaine have the property of maintenance of glycolysis over and above the normal velocity of this process in an activated homogenate of rat brain. During the first ten minutes, there is no difference in the rate of glycolysis. However, after the first ten minutes, the normal rate tends to decrease, while the mixture containing either of these two drugs maintains the same rate of the original ten minute period. Using radioactives phosphorous in the form of the phosphate ion, Pertzoff and Gemmill<sup>46</sup> have shown that sodium barbital and ether have a retarding effect on the transfer of phosphate from plasma into the red blood cell.

#### SUMMARY

Several examples of the action of chemical compounds of therapeutic interest on glycolysis have been given in this short review. In most of these cases, the methods and results of Professor Meyerhof have been used as a background in this work. Many developments are possible from this type of work, especially in the explanation of drug action and the control of disease through this knowledge. Therefore, pharmacology owes much to the pioneer investigations of Professor Meyerhof.

#### RÉSUMÉ

Dans cette brève revue nous avons donné plusieurs exemples de l'action sur la glycolyse de certains composés chimiques d'intérêt pharmaceutique. Dans la plupart des cas les méthodes et les résultats du Professeur Meyerhof ont formé le point de départ de ce travail. Ce genre de travail peut donner lieu à des développements nombreux, surtout pour expliquer l'action des drogues et, par ce fait, pour enrayer la maladie. C'est pourquoi la pharmacologie doit beaucoup aux investigations de pionnier du Professeur Meyerhof.

#### ZUSAMMENFASSUNG

In dieser kurzen Übersicht wurden einige Beispiele für die Wirkung chemischer Verbindungen von therapeutischem Interesse auf die Glykolyse gegeben. In den meisten Fällen bildeten die Methoden und die Ergebnisse von Professor Meyerhof den Hintergrund dieser Arbeit. Vielerlei Entwicklungen dieser Arbeit sind möglich, insbesondere zur Erklärung der Wirkung der Arzneimittel und dadurch zur Eindämmung der Krankheiten. Deshalb hat die Pharmakologie den Pioniersuntersuchungen von Professor Meyerhof viel zu verdanken.

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# ZUR CHARAKTERISIERUNG DER SPEZIFITÄT PHARMAKOLOGISCHER WIRKUNGEN UND DES SIE BEDINGENDEN REZEPTORSYSTEMS DES SUBSTRATES

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Die Arbeiten von Otto Meyerhof haben im wesentlichen die Analyse physiologischer Reaktionen zum Ziele, besonders die quantitative Feststellung des Reaktionsablaufes, seiner Bedingungen und seiner Gleichgewichtszustände. Seine Auffassung der Dynamik der physiologischen Vorgänge hat zu den — Grundlage einer Arbeitsrichtung gewordenen — Ergebnissen geführt. Auch für andere Disciplinen haben diese Untersuchungen grundsätzliche Bedeutung gewonnen, so auch für die Pharmakologie, für die gerade die quantitative Analyse der physiologischen Reaktionen einen besonderen Zugang zu ihrem eigentlichen Problem der Analyse der Wirkung von Arzneimitteln eröffnet hat. Im allgemeinen ist es allerdings in vielen Fällen heute noch nicht möglich, die pharmakologische Wirkung auf die Reaktionsteilnehmer physiologischer Reaktionen zurückzuführen. In der weitaus grösseren Zahl der Fälle ist die Beteiligung des zugesetzten Pharmakons am ausgelösten Prozess nicht bekannt, sodass sich die pharmakologische Feststellung sehr häufig zunächst damit begnügen muss, aus der quantitativen Ermittlung des durch zugesetzte Pharmaka ausgelösten Reaktionsverlaufes zu einer praeliminären Charakterisierung des zugrundeliegenden Vorganges zu kommen. Besonders die Beziehung zwischen der gegebenen Dosis und dem eintretenden Effekt ist Gegenstand der Analyse des Vorganges geworden. Die Forschungen von Loewe (1928), CLARK (1933, 1937) und GADDUM (1937) haben vor allem allgemeingültige Folgerungen an der Bewertung derartiger Befunde entwickelt. In Parallele zur mathematischen Behandlung chemischer und physikalisch-chemischer Reaktionen war es naheliegend, die gleichen Prinzipien auch auf die Reaktionen von Pharmaka anzuwenden. Clark hat um die Behandlung biologischer Daten in dieser Richtung die grössten Verdienste. Es lässt sich aber für diese Art der Analyse die Schwierigkeit nicht eliminieren, inwieweit die Dosiswirkungsbeziehung allein oder auch nur im wesentlichen durch die Reaktion des Pharmakons mit dem spezifischen Rezeptor bedingt ist, da die Beteiligung des Pharmakons an einem bestimmten Vorgange, die Reaktion desselben mit einem bestimmten Reaktionsobjekt in der Zelle oder auch ein Reaktionsprodukt dieses Vorganges im allgemeinen noch nicht exakt festgestellt wer-

Es soll in dieser Mitteilung nicht zu den sich hier ergebenden Problemen allgemein Stellung genommen werden, sondern nur eine Frage aus diesem Zusammenhang behandelt werden. Ein besonders wichtiges, vielleicht das wesentlichste Problem der Literatur S. 154/155.

Pharmakologie ist die Erforschung der Ursache der Spezifität pharmakologischer Wirkungen, da bestimmte differenzierte Wirkungen eben nur dadurch möglich werden, dass eine Substanz mit einer wesentlich niedrigeren Konzentration an einem bevorzugten Reaktionsort zu wirken vermag. Die zu behandelnde Frage wäre so zu umreissen: Lassen sich quantitative Beziehungen des Reaktionsverhaltens eines biologischen Objektes auffinden, welche mit der Spezifität der Wirkung in einem direkten Zusammenhang stehen, und welche Befunde lassen Schlussfolgerungen auf die Art der Reaktion des spezifischen Reaktionssystemes des biologischen Objektes zu?

Im allgemeinen liegen nicht genügend Untersuchungen vor, welche das Reaktionsverhalten von Substanzen mit hoher Spezifität und anderen Angehörigen der gleichen Gruppe mit wesentlich geringerer Spezifität unter gleichen Bedingungen feststellen. Ferner werden häufig Befunde an verschiedenen Objekten untereinander verglichen. Dies liegt zum Teil in der Natur der Objekte, weil nur in Ausnahmefällen Reaktionen verschiedenen pharmakologischen Charakters in gleicher Weise am gleichen Objekt untersucht werden können. Alle diese Momente bieten Unsicherheiten für die Beurteilung. In den letzten Jahren wurde in unseren Laboratorien eine interessante Gruppe pharmakologischer Körper bearbeitet, welche für die Untersuchung der genannten Fragen gewisse Vorteile bietet, die Gruppe der aromatischen Imidazolinderivate. Diese chemische Struktur hat die besondere Eigenschaft, dass durch entsprechende chemische Abwandlung in dieser Gruppe Stoffe mit verschiedenartigsten Wirkungen sehr hoher Spezifität entwickelt werden können. Es finden sich in ihr neben Sympathikomimetika Sympathikolytika, neben Antihistaminen histaminergische Stoffe, ausserdem Parasympathikolytika und Parasympathikomimetika und andere Stoffe hoher Spezifität. Es muss somit in dieser Struktur eine eigenartige potentielle Möglichkeit zur Reaktion mit den verschiedenen Wirkorten des biologischen Substrates enthalten sein, da eine Gruppe von chemischen Verbindungen vorliegt, die bei prinzipiell gleichartiger Grundstruktur sehr viele verschiedenartige Wirkungsqualitäten aufweist (MEIER, 1947). Es können somit Dosiswirkungskurven von Stoffen gleichartiger chemischer Struktur mit verschiedenem Spezifitätsgrad und verschiedenartigem Wirkungscharakter mit einander verglichen werden.

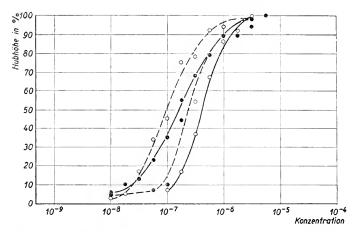
Die erste zu behandelnde Frage ist die: Bestehen zwischen der Dosiswirkungsbeziehung und der Spezifität der Wirkung Beziehungen allgemeineren Charakters? In der Literatur werden eine Reihe von Angaben über diese Möglichkeit gegeben und zum Teil ziemlich weitgehende Aussagen über die Bedeutung bestimmter Verlaufsformen der Dosiswirkungskurve für bestimmte Wirkstoffgruppen in Anspruch genommen (Storm van Leeuwen, 1919, Clark, 1937).

Auf Grund unserei Untersuchungen, in denen verschiedenartigste Stoffe in dieser Hinsicht untersucht wurden, geht nicht hervor, dass sich in Veränderungen der Dosiswirkungskurven Gesetzmässigkeiten finden, welche direkt mit der Spezifität der Wirkung in Zusammenhang stehen. Man erkennt in Abbildung i und 2, dass sich an der isolierten Samenblase des Meerschweinchens und an der isoliert durchströmten Hinterextremität des Kaninchens die Dosiswirkungskurven verschiedener Wirkstoffe und von verschiedener oder gleicher chemischer Struktur formal weitgehend ähnlich verhalten.

Die Dosiswirkungsbeziehungen des Otrivins an der Meerschweinchen-Samenblase und am Meerschweinchen-Dünndarm sind formal ebenfalls praktisch identisch, trotzdem Otrivin an der Samenblase etwa die gleiche oder eher ausgesprochenere Wirkungs-

Abb. I. Isolierte Meerschweinchen-Samenblase. Dosiswirkungskurven von Adrenalin, Otrivin, Acetylcholin und Histamin. Abszisse: Konzentrationen (logarithmisch).

Ordinate: Hubhöhe in Prozent (numerisch) (Wirsing, 1949).



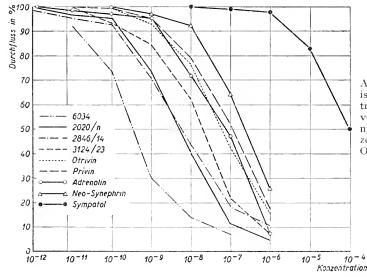
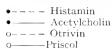
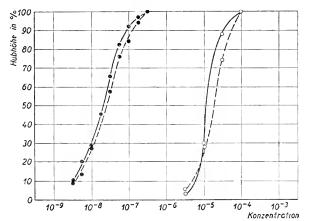


Abb. 2. Gefässdurchfluss der isolierten Kaninchenhinterextremität. Dosiswirkungskurven von 6 Imidazolinen und 3 Phenyläthylaminen. Abszisse: Konzentrationen (logarithmisch). Ordinate: Durchfluss in Prozent (numerisch) (Meier und Pellmont, 1947).

Abb. 3. Isolierter Meerschweinehen-Dünndarm. Dosiswirkungskurven von Histamin, Acetylcholin, Otrivin und Priscol. Abszisse: Konzentrationen (logarithmisch). Ordinate: Hubhöhe in Prozent (numerisch).





stärke besitzt als Acetylcholin oder Histamin, während es am Dünndarm rund 500 mal schwächer wirksam ist (Abb. 3).

Naturgemäss bleibt ein wichtiges Moment bei diesen Untersuchungen verborgen: Die Konzentration der Wirkstoffe an den Reaktionsorten. Es kann wohl ausgeschlossen werden, dass nicht genügend starke Konzentrationen an die Reaktionsorte der Zelle gelangen. Da im allgemeinen diese Stoffe für eine andere Reaktion eine hohe Spezifität besitzen und diese ohne weiteres ausgelöst werden kann, ist es nicht wahrscheinlich, dass ein wesentlich kleinerer Prozentsatz des zugesetzten Stoffes in die Zelle hineingelangt. Immerhin ist diese Möglichkeit nicht vollständig auszuschliessen. Vollständig unbekannt ist aber, in welchem Umfange sich der Stoff innerhalb der Zelle zwischen spezifischen und unspezifischen Reaktionsorten verteilt. Wenn man annimmt, dass in einem physikalisch-chemischen Ablauf der Reaktion zwischen den spezifischen und unspezifischen Reaktionsorten der Zelle kein wesentlicher Unterschied besteht und die Spezifität der Wirkung ausschliesslich auf einer Verteilung zwischen diesen verschiedenen Reaktionsorten beruht, ist es durchaus möglich, dass nur diese Unterschiede der Verteilung die Ursache des Spezifitätsgrades darstellen. Es muss somit auf Grund dieser Untersuchungen geschlossen werden, dass zwischen der Spezifität oder dem spezifischen Charakter einer Wirkung und der Dosiswirkungsbeziehung kein direkter Zusammenhang besteht. Wir glauben deshalb, in der Interpretation solcher Dosiswirkungsbeziehungen auf Grund unserer heutigen Kenntnisse den Erklärungsversuchen von Gaddum (1926, 1937) folgen zu können, der mit Shackell (1925) und Fromherz (1926) annimmt, dass Konzentrationswirkungskurven lediglich die Wirkung eines Giftes an einer Zellpopulation zum Ausdruck bringen, d.h. die durch eine bestimmte Dosis hervorgerufene Wirkung wäre eine Resultante der Wirkung einzelner aktiver Elemente, die gegenüber einem einwirkenden Agens verschieden empfindlich sind, wobei unter den aktiven Elementen ganze Zellen oder nur Teile solcher, wie Rezeptoren, angenommen werden könnten.

Eine besonders viel gebrauchte Art der Charakterisierung pharmakologischer Reaktionen ist in den letzten Jahren die Untersuchung von antagonistischen Wirkungen geworden. "Antagonisten" besitzen im allgemeinen keine Eigenwirkung auf das Substrat, vermögen aber die durch einen bestimmten Agonisten hervorgerufene Reaktion eines Substrates in spezifischer Weise zu verhindern. Es besteht somit die Möglichkeit, dass bei diesen Stoffen eine besonders günstige Situation gegeben ist, um das quantitative Reaktionsverhalten von pharmakologischen Mechanismen zu untersuchen. Es wurden im wesentlichen die gleichen Untersuchungen wie für die eingangs besprochenen Agonisten ausgeführt. Es soll verzichtet werden, auf die Befunde der Literatur im einzelnen einzugehen. Für diese Gruppe sind die Imidazolinderivate bezonders geeignet, weil sich — wie eingangs erwähnt — ausser primär wirkenden Stoffen wie Sympathikomimetika, histaminergische Stoffe, auch antagonistische Stoffe hoher Spezifität in dieser chemischen Gruppe finden. Es ergibt sich, dass antagonistisch wirkende Stoffe, welche einer im wesentlichen gleichen Grundstruktun der aromatischen Imidazoline zugehören, aber von sehr verschieden hohem Spezifitätsgrad sind, im wesentlichen einen gleichartigen Verlauf der Dosiswirkungskurve zeigen. Weiterhin ist festzustellen, dass dieses nicht nur der Fall ist bei einem spezifischen Vorgang, wie z.B. dem Antagonismus der Sympathikolytika gegenüber den Sympathikomimetika, sondern dass auch bei den übrigen Reaktionen hoher Spezifität wie dem Antagonismus gegen Histamin oder dem Antagonismus gegen Acetylcholin ein weitgehend uniformes Verhalten der Dosiswirkungsbeziehung antagonistisch wirkender Stoffe vorliegt. Eine Sonderstellung scheint

unter den bisher untersuchten antagonistischen Reaktionen am Meerschweinchen-Dünndarm lediglich dem Antagonistenpaar Acetylcholin-Adrenalin zuzukommen (Bein, 1947), wobei die Frage nach der Ursache dieser Verschiedenheit heute noch offen gelassen werden muss. Möglicherweise könnte dieses unterschiedliche Verhalten dadurch bedingt sein, dass es sich bei dieser Stoffkombination um einen "funktionellen Antagonismus" handeln würde.

Da nicht nur eine Spezifitätshöhe der Wirkung im Vergleich verschiedener chemischer Stoffe, sondern auch eine verschieden hohe Spezifität der Wirkung gegenüber einer gegebenen Skala von verschiedenen Reaktionsobjekten besteht, sind auch die Dosiswirkungsbeziehungen an verschiedenen Objekten zu untersuchen. Es sind, wie bereits erwähnt, nun nicht sehr viele Objekte vorhanden, an denen derartige Untersuchungen für alle möglichen Fälle durchgeführt werden können. Immerhin haben wir eine Reihe von Beispielen aus diesen bereits besprochenen Stoffgruppen in der Weise untersucht, dass sowohl die Dosiswirkungskurve von agonistischen und antagonistischen Wirkungen sowohl an der Samenblase (Abb. 4) wie dem isolierten Dünndarm des Meerschweinchens (Abb. 5 und 6) und zum Teil auch am Froschherzen und einzelnen anderen Objekten aufgestellt wurden. Das Untersuchungsmaterial, welches in dieser Hinsicht vorliegt, ist nicht so vollständig wie es wünschenswert wäre. Es ergibt sich, dass sowohl bei der Verwendung von Agonisten verschiedener chemischer Struktur als auch verschiedener Wirkungsrichtung Dosiswirkungskurven erhalten werden können, die für das eine Objekt einen etwas anderen Wirkungstypus besitzen wie für ein anderes. Im allgemeinen sind die Dosiswirkungskurven nicht bedingt durch den verschiedenen Spezifitätsgrad der Wirkung an diesen verschiedenen Objekten, sondern die Dosiswirkungskurven an einem Objekt pflegen im allgemeinen einem bestimmten Typus zu folgen, während sie an einem anderen Objekt einen anderen Typus besitzen. Aus Abb. I geht hervor, dass beim Meerschweinchen die isolierte Samenblase — ähnlich dem isolierten Uterus (Fromherz, 1926) und im Gegensatz zum isolierten Dünndarm (Abb. 3) — die Tendenz zeigt, bei verschiedenen Konzentrationen von unterschiedlich wirksamen Stoffen bald ein Maximum der Antwort zu erreichen, wenn auch an der Samenblase wahrscheinlich besser als beim Uterus in dieser Hinsicht noch eine gewisse Differenzierung zwischen einzelnen Pharmaka durchgeführt werden kann. Auch die absolute Wirkungsstärke von antagonistisch wirkenden Stoffen kann an verschiedenen Objekten stark wechseln, so braucht es z.B. am isolierten Meerschweinchen-Dünndarm etwa fünfmal mehr Antistin, um eine gegebene Histaminkontraktur zu unterdrücken, während an der isolierten Meerschweinchen-Samenblase dieses Verhältnis gerade umgekehrt ist. Es scheint somit, dass nicht die Spezifität der Wirkung, sondern eine Eigentümlichkeit des Substrates im Verhältnis zur untersuchten Stoffgruppe eine unterschiedliche Dosiswirkungsbeziehung bedingt. Ganz ähnlich, jedenfalls durchaus nicht grundsätzlich anders liegen die Verhältnisse für die antagonistischen Reaktionen.

Die Feststellung der Dosiswirkungsbeziehung antagonistischer Reaktionen bietet noch eine besondere Möglichkeit für die quantitative Feststellung des Reaktionsverhaltens, die bisher von verschiedenen Seiten benutzt wurde. Es kann festgestellt werden, ob bei Steigerung der Konzentration des Agonisten auch eine relativ gleichstarke Steigerung des Antagonisten zu erfolgen hat, woraus geschlossen werden könnte, dass zwischen der Affinität des Agonisten und des Antagonisten unabhängig von der Konzentration des Agonisten die gleiche Reaktionsbereitschaft besteht. Auch ein derartiges Verhalten könnte naturgemäss Anhaltspunkte für die Spezifität einer Reaktion geben.

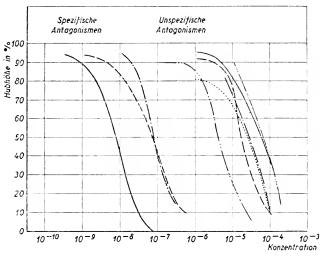


Abb. 4. Isolierte Meerschweinchen-Samenblase. Dosiswirkungskurven verschiedener Stoffkombinationen. Abszisse: Konzentrationen der Antagonisten (logarithmisch). Ordinate: Hubhöhe in Prozent (numerisch) (WIRSING, 1949).

Spezifische Antagonisten:

Atropin/Acetylcholin
Antistin/Histamin
7337n/Adrenalin

Unspezifische Antagonismen:

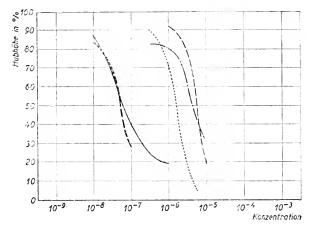
Priscol/Adrenalin
Atropin/Adrenalin
Atropin/Adrenalin
Antistin/Adrenalin
Atropin/Histamin

····· 7337 n/Histamin
-··- Antistin/Acetylcholin

Abb. 5. Isolierter Meerschweinchen-Dünndarm. Dosiswirkungskurven verschiedener Histamin-Antagonisten bei einer gegebenen Histaminkonzentration von 10<sup>-7</sup>.

Abszisse: Konzentrationen der Antagonisten (logarithmisch)

Ordinate: Hubhöhe in Prozent (numerisch) (MEIER, 1947)



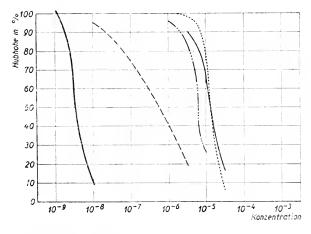


Abb. 6. Isolierter Meerschweinchen-Dünndarm. Dosiswirkungskurven verschiedener Acetylcholin-Antagonisten bei einer gegebenen Acetylcholinkonzentration von 10<sup>-7</sup>. Abszisse: Konzentrationen der Antagonisten (logarithmisch). Ordinate: Hubhöhe in Prozent (numerisch) (Meier, 1947).

Literatur S. 154/155.

Bereits früheren Untersuchern ist es aufgefallen, dass besonders mit relativ geringen Dosen eines Agonisten oder eines Antagonisten eine solche Gesetzmässigkeit dieser Relation nicht beobachtet werden kann. So muss, um nur ein Beispiel zu erwähnen, am isolierten Kaninchendarm bei einer Erhöhung der Pilocarpinkonzentration die für einen gleichen Effekt notwendige Atropindosis um nur wenig mehr erhöht werden, (MAGNUS, 1908), während umgekehrt am isolierten, elektrisch gereizten Ventrikelstreifen des Frosches in einem niedrigen Dosenbereich verhältnismässig mehr Atropin als Acetylcholin für einen konstanten Effekt gegeben werden muss (Clark, 1926). In eigenen Versuchen, in welchen wir am isolierten Meerschweinchen-Dünndarm sowohl die Konzentration von Agonisten, Histamin und Acetylcholin, als auch diejenige von Antagonisten, Pyribenzamin, Neo-Antergan und Antistin, resp. Atropin und Trasentin steigerten, ergab sich ebenfalls ein inkonstantes Verhältnis. Merkwürdigerweise scheint hier unter den gewählten Versuchsbedingungen (Einwirkungsdauer der Antagonisten jeweils 2 Minuten) bei hochwirksamen Antagonisten (Pyribenzamin, Neo-Antergan, Atropin) eine relativ kleinere Dosissteigerung notwendig zu sein als bei etwas weniger wirksamen (Antistin, Trasentin). Auf der anderen Seite muss bei den unspezifischen antagonistischen Reaktionen Neo-Antergan-Acetylcholin und Pyribenzamin-Acetylcholin bei 10-facher Steigerung der Acetylcholinkonzentration die Konzentration der Antagonisten für einen gleichen Effekt ebenfalls nur um wenig mehr erhöht werden.

Wenn auch bei gleichzeitiger Steigerung sowohl einer Agonisten- wie auch einer Antagonistenkonzentration das gegenseitige Mengenverhältnis, das auch beim gleichen Antagonistenpaar für verschiedene Objekte variiert, durch eine mathematische Beziehung ausgedrückt werden kann (Clark, 1926, 1937; Gaddum, 1937), so bleibt doch die Schwierigkeit der gedanklichen Vorstellung. Guzman Barron und Mitarbeiter (1948) haben kürzlich gezeigt, dass in einer Zelle zwei verschiedenartige Sulfhydrilgruppen angenommen werden können, die mit SH-Gruppen blockierenden Giften je nach deren Konzentration reagieren. Entsprechend dieser Vorstellung könnten zwei oder mehrere Rezeptorengruppen angenommen werden, die sich gegenüber einem Agonisten wie auch gegenüber einer antagonistisch wirkenden Substanz verschieden empfindlich verhalten. Das gegenseitige Mengenverhältnis Agonist/Antagonist bei jeweils steigenden Konzentrationen würde dann aus einer Resultante der Wirkung an den verschiedenen Rezeptorengruppen stammen.

Es scheint somit, so interessant diese Untersuchungen sind, und so interessant sie für die Feststellung der relativen Affinität zu gewissen Reaktionsorten der Zelle sind, dass sie offenbar die Spezifitätshöhe der pharmakologischen Wirkung nicht direkt begründen können, wobei naturgemäss wieder als eine Vermutung nahegelegt wird, dass tatsächlich die Dosiswirkungskurve nicht nur ein Ausdruck der spezifischen, sondern auch unspezifischer Reaktionsorte der Zelle sein mag.

Wenn auch mit der Feststellung der spezifischen Hemmbarkeit eines oder verschiedener Agonisten durch einen Antagonisten ein gemeinsamer Angriffspunkt postuliert werden kann, so braucht nun der Wirkungsablauf der verschiedenen Agonisten noch nicht gleich zu sein, da einerseits eine antagonistisch wirkende Substanz eine Reaktionskette, von welcher wir meist nur die Endreaktion beobachten, an jeweils verschiedenen Stellen unterbrechen kann, oder weil anderseits die Reaktionskette von einem primären Ausgangspunkt an verschieden verläuft.

Ein weiteres Vorgehen besteht in der Feststellung der Zeitwirkungskurve, die im Prinzip wohl der Erreichung der Einstellung eines Reaktionsgleichgewichtes zwischen Literatur S. 154/155.

dem der Lösung zugesetzten Wirkstoff und den Reaktionsorten der Zelle angesehen werden kann. Dass bei dieser Untersuchung die Permeabilitätsfrage eine wesentliche Bedeutung besitzt, ist ohne weiteres auf der Hand liegend, und es muss jedenfalls diese Möglichkeit bevor ein Urteil über die Reaktion mit spezifischem Ort in der Zelle hier in Anspruch genommen wird, im Auge behalten werden. Immerhin kann aber auch eine solch "unspezifische" Reaktion wie die Veränderung der Permeabilität ebenfalls ein für einen Wirkstoff bis zu einem gewissen Grade charakteristisches Verhalten darstellen. Als besonders entscheidend müssen wieder diejenigen Untersuchungen angesehen werden, bei denen die Zeitwirkungskurve von Imidazolinvertretern gleicher chemischer Grundstruktur aber verschiedener Wirkungsspezifität angesehen werden. Es wäre an und für sich möglich, dass bei diesen die Eintrittszeiten verschieden sind, weil naturgemäss bei Stoffen verschieden hoher Spezifität die Aussenkonzentrationen verschiedenartig sind, je nach der Wirkungshöhe des untersuchten Stoffes. Wenn auch gewisse Unterschiede bei Stoffen verschiedener chemischer Struktur hinsichtlich des Eintrittes

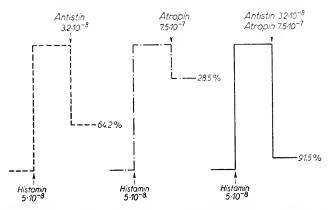


Abb. 7. Isolierter Meerschweinchen-Dünndarm. Einfache Addition der Wirkung von zwei verschiedenen antagonistisch wirkenden Stoffen (Mittelwerte aus 5 Versuchen).

des Reaktionsgleichgewichtes vorhanden sind, muss man doch sagen, dass bei den Imidazolinderivaten mit sehr unterschiedlicher Spezifität der Wirkung keine typischen Unterschiede zu beobachten sind, die dafür sprechen, dass die Geschwindigkeit der Reaktion mit den für die Wirkung verantwortlichen Reaktionsorten der Zelle in einem direkten Zusammenhang mit der Spezifitätshöhe der Wirkung steht.

Es bleiben, darauf muss hingewiesen werden, gewisse Unterschiede sowohl der Dosiswirkungskurven wie der Zeitwirkungskurven bestehen. Die Abweichungen dieser Befunde liegen aber relativ so nahe in der Fehlerbreite der Untersuchungsmethoden, dass es verfrüht erscheint, diese Abweichungen zum Gegenstand allgemeiner Schlussfolgerungen zu machen. Sie bedürfen sicher weiterer Aufmerksamkeit und es scheint möglich, dass ihnen für die Beurteilung des spezifischen Reaktionsverhaltens noch eine grössere Bedeutung zukommen wird.

Da am isolierten Kaninchenuterus die volle Hemmwirkung z.B. des Ergotamins gegenüber Adrenalin erst nach Stunden eintritt (GADDUM, 1926), am Kaninchendarm jedoch schon nach Minuten (ROTHLIN, 1929), so könnte es auch sein, dass eine solche Zeitmessung nicht einen Vorgang erfasst, der sich an den Rezeptoren selbst abspielt, sondern nur ein mehr oder weniger rasches Durchwandern durch das Gewebe zu den

aktiven Gruppen (Gaddum, 1937). Es ist jedoch mit dieser Annahme schwer zu vereinbaren, warum das Adrenalin, dessen Wirkungseintritt, d.h. dessen Verbindung mit seinen aktiven Rezeptoren, innerhalb von wenigen Sekunden erfolgt, am isolierten Meerschweinchen-Dünndarm seine maximale antagonistische Wirkung gegenüber Acetylcholin auch nach 10 Minuten noch nicht erreicht hat (Vest), wenn nicht angenommen wird, dass durch eine antagonistisch wirkende Substanz nicht nur "Rezeptoren blockiert", sondern möglicherweise auch gleichzeitig andere Prozesse wie z.B. die Permeabilität oder Stoffwechselvorgänge und anderes mehr, verändert werden müssten. Dass in diesem Zusammenhang auch der Frage der Haftfestigkeit eine Bedeutung zukommt, braucht wohl nicht näher ausgeführt zu werden.

Hinsichtlich der eingangs gestellten Frage des Zusammenhanges quantitativer Reaktionsverhältnisse mit der Spezifitätshöhe pharmakologischer Reaktionen muss somit gesagt werden, dass die bisher von uns durchgeführten Untersuchungen keinen Anhaltspunkt dafür geben, dass dieser quantitative Reaktionsverlauf in irgendeiner Weise zur Erklärung der Spezifitätshöhe herangezogen werden kann. An dem Substrat, an dem sich die spezifischen Reaktionen abspielen, können sich die spezifischen und unspezifischen Reaktionen an sich nur dadurch unterscheiden, dass in der Verteilung innerhalb verschiedenartiger Reaktionsorte in der Zelle die spezifischen Wirkstoffe bevorzugt die spezifischen Reaktionsorte erreichen, auch wenn in der Aussenflüssigkeit und vielleicht auch in der Zelle und an unspezifischen Reaktionsorten eine höhere Konzentration der letzteren vorhanden ist. Es wird bei dieser Sachlage naturgemäss schwierig, hinsichtlich der spezifischen Reaktion allgemein verbindliche Schlussfolgerungen zu ziehen, da auch angenommen werden kann, dass bei einem wesentlichen Teil der Reaktion mit unspezifischen Reaktionsorten der Zelle das gesamte Verhalten der Dosiswirkungsbeziehung durch die unspezifische Reaktion mitbedingt sein kann. Es ist besonders wichtig zu entscheiden, ob tatsächlich alle die ausgelösten Reaktionen eine Wirkung am gleichen Reaktionsort hervorrufen, oder ob nicht noch andere indirekte Wirkungsmöglichkeiten vorhanden sein können, welche den Eindruck einer Wirkung am gleichen Reaktionsort besitzen, trotzdem sie eigentlich nicht als "spezifischer" Antagonismus im eigentlichen Sinne aufzufassen sind. Für die Entscheidung dieser Frage ist es von ausschlaggebender Bedeutung, die Separation der spezifischen Reaktionen nachzuweisen. Das hier meistens angewandte Verfahren, welches in einfacher Weise einen solchen "Spezifitätsgrad" der Wirkung beweist, ist dasjenige, dass die Wirkung eines Stoffes an einem bestimmten Reaktionssystem z.B. am histaminergischen System, untersucht wird, während die Reaktion des parasympathischen Systems durch Atropin ausgeschaltet wird. Auch dann, wenn an diesem System keinerlei Wirkung durch eine gegebene Dosis Acetylcholin mehr ausgelöst wird, kann mit einem anderen Stimulans z.B. Histamin, die entsprechende Reaktion in gleicher Weise ausgelöst werden. Dieses spricht selbstverständlich ohne weiteres dafür, dass eine Differenziertheit der Substrate vorhanden ist. Unterlagen hinsichtlich der Dosiswirkungsbeziehung verschiedenartiger Stoffe mit verschiedenartiger Spezifität unter derartigen Bedingungen sind allerdings nicht vorhanden. Eine weitere Möglichkeit besteht in der Verwendung der Addition verschiedenartiger spezifischer Effekte. Ein hierfür zweckmässiges Verfahren ist die Auslösung von je 50% des Maximaleffektes durch je einen Agonisten, z.B. Histamin oder Acetylcholin am Meerschweinchendarm. Bereits die additive Wirkung von derartigen Dosen zeigt, dass eine besondere Differenzierung zwischen dem Reaktionsort und dem Kontraktionssubstrat vorhanden sein muss, der bewirkt, dass der

Effekt verschiedenartiger Stimulantien eine einfache Summation des Einzeleffektes am Erfolgsorgan ergibt. Diese Feststellung bietet gewisse Schwierigkeiten für die Erklärung mancher antagonistischer Wirkungen, bietet aber auch die Möglichkeit, den hohen Spezifitätsgrad antagonistischer Wirkungen nachzuweisen. Bringt man z.B. einen isolierten Darm mit Dosen, welche jeweils eine 50% ige Kontraktion der maximalen Histamin- und Acetylcholin-Kontraktur bewirken, zur Kontraktion, so tritt eine 100% ige Kontraktur, wie bereits eben besprochen wurde, ein. Wendet man nun diejenigen Konzentrationen der Antagonisten, z.B. Atropin und Antistin, an, welche gerade 50% der Wirkung zum Verschwinden bringen, so tritt auch in diesem Falle nur eine Aufhebung des durch den entsprechenden Agonisten hervorgerufenen Effektes auf, was wiederum beweist, dass eine Separation der Angriffspunkte sowohl der agonistischen als auch der antagonistischen Wirkung vorhanden ist. Diese Befunde sprechen wohl dafür, dass ein Verdrängungsvorgang für die antagonistische Reaktion von Bedeutung ist. Wenn nun eine Separation der Angriffspunkte der spezifischen Agonisten vorhanden ist, sowohl untereinander als auch hinsichtlich des von ihnen bewirkten Substrates, so lassen sich doch aus diesen Befunden keine weiteren Argumente für die Struktur des spezifischen Substrates erhalten.

Es gibt aber noch eine Möglichkeit, welche vielleicht etwas weiteren Aufschluss über die Separation der Wirkorte ergeben kann. Es sind Stoffe bekannt geworden, welche im gleichen Molekül zwei spezifische Wirkungen besitzen, z.B. sympathikolytische und histaminolytische Wirksamkeit, atropinartige und histaminolytische und so fort. Nur ausnahmsweise gelingt es, Stoffe zu erhalten, bei denen die Wirkungshöhe dieser beiden Wirkungsqualitäten von absolut gleicher Stärke vorhanden ist. Es lässt sich nun mit Hilfe dieser Stoffe folgende Frage beantworten. Bewirkt ein derartiger Stoff wie z.B. Vertreter der Tetrahydrofluoranthene eine antagonistische Reaktion z.B. gegenüber Histamin und Acetylcholin, so fragt es sich, ob bei jeweils 50% iger Kontraktion durch Histamin und 50% iger Kontraktion durch Acetylcholin eine Konzentration des Stoffes gebraucht wird, welche die 100%ige Lyse der Acetylcholin- oder der Histaminkontraktur hervorruft, oder ob für die Aufhebung dieses Effektes eine Konzentration genügt, welche 50% antagonistisch beeinflusst. Es stellt sich bei der Untersuchung dieser Frage heraus, dass in der Tat für die Aufhebung einer summierten Kontraktion aus 50% Histamin- und 50% Acetylcholinkontraktur nicht diejenige Konzentration gebraucht wird, welche die Maximalkontraktion mit Histamin, bzw. Acetylcholin löst, sondern dass nur diejenige Konzentration des Stoffes nötig ist, welche eine jeweils 50% ige Wirkung aufzuheben imstande ist. Für dieses eigenartige Verhalten könnten vor allem zwei verschiedene Möglichkeiten in Anspruch genommen werden, nämlich dass die vorhandene Menge des antagonistisch wirkenden Stoffes, trotzdem er nur mit 50% des Histaminreaktionssubstrates antagonistisch reagiert, auch gleichzeitig mit 50% des Acetylcholinsubstrates reagiert, wobei diese beiden Substrate als separiert von gleicher Empfindlichkeit gedacht sind. Die zweite Möglichkeit wäre diejenige, dass das gleiche Molekül des antagonistisch wirkenden Stoffes gleichzeitig mit dem Acetylcholin- als auch mit dem Histaminrezeptor reagiert. Wäre dies der Fall, so würde daraus zu schliessen sein, dass strukturchemisch die Angriffsorte des Histamins und Acetylcholins räumlich so nahe beieinander gelagert sind, dass ein Molekül des Antagonisten beide gleichzeitig beeinflussen kann. Es lässt sich zwischen diesen beiden Möglichkeiten vorläufig nicht entscheiden; es sind weitere Untersuchungen in dieser Richtung im Gange und es ist nicht vollständig ausgeschlossen dass sich Argumente für die letztere Möglichkeit

werden beibringen lassen. Der Nachweis der funktionellen Separation der Rezeptionsorte der Zelle für die spezifische Reaktion gibt die Möglichkeit, eine Reihe von Eigenschaften dieses Reaktionssubstrates auf Grund der eingangs besprochenen Untersuchungen aufzustellen: Das Reaktionssubstrat muss in der Lage sein, mit hoher Spezifität mit Stoffen verschiedenartiger chemischer Grundstruktur so zu reagieren, dass ihnen der gleiche Wirkungscharakter zukommt. Das Substrat muss mit Stoffen grundsätzlich gleichartiger chemischer Struktur so reagieren können, dass nur einzelne, die in bestimmter Weise substituiert sind, die höchste Spezifität besitzen, und die Reaktionsorte verschiedenartigen Wirkungscharakters sind imstande, Stoffen gleichartiger chemischer Grundstruktur, die sich nur durch bestimmte Substituenten voneinander unterscheiden, die spezifische Reaktion zu erlauben. Zum Teil lassen sich diese Eigentümlichkeiten des Reaktionssubstrates durch die Wirkung der Agonisten finden, zum Teil haben sie nur für die Wirkung von Antagonisten Geltung, weil nur mit Hilfe dieser das entsprechende Verhalten bisher nachgewiesen werden konnte. Die Organisation des empfindlichen Substrates ist nicht dadurch gekennzeichnet, dass quantitative Einstellungen des Reaktionsgleichgewichtes die Ursache der unterschiedlichen Spezifität der Wirkung sind. Ebenso ist für die Spezifität der Reaktion nicht die relative Empfindlichkeit gegenüber Agonisten oder Antagonisten direkt verantwortlich. Diese verschiedenen Eigentümlichkeiten des Reaktionssubstrates und damit auch die Eigenschaften, welche für die Spezifität der pharmakologischen Wirkung verantwortlich sind, lassen sich am einfachsten so erklären, dass für die Spezifität der Wirkung eine bestimmte chemische oder physikalische Struktur des Substrates verantwortlich ist. Da dieses Substrat ganz bestimmte eigentümliche Eigenschaften besitzen muss, kann nur dann eine Reaktion an einem Substrat als Erklärung oder als Analogon dieses Reaktionsverhaltens der Zelle in Anspruch genommen werden, wenn dieses Substrat de facto sämtliche Eigenschaften besitzt, welche im vorstehenden auf Grund der quantitativen Reaktionsverhältnisse festgestellt wurden. Wenn somit diese Untersuchung nicht die Frage der Zurückführung der Wirkungsspezifität auf allgemeine physikalische oder chemische Gesetzmässigkeiten behandelte, so kann die quantitative Analyse derartiger Reaktionsgleichgewichte doch dazu beitragen, einfachere Modelle als identisch oder nicht identisch mit dem Substrate der pharmakologischen Wirkung zu bezeichnen oder nicht. Dieses dürfte wohl einer der Wege sein, auf dem versucht werden kann, die Komplexität des pharmakologischen Reaktionsverhaltens in seine einzelnen Elemente aufzulösen.

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#### PART IV

#### INTERMEDIATE METABOLISM

### FREE RADICALS DERIVED FROM TOCOPHEROL AND RELATED SUBSTANCES

by

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Tocopherol is known to exhibit two properties: It serves as a vitamin, and also as an antioxidant with respect to the autoxidation of unsaturated fatty acids. The latter property is shared with many substances of phenolic character. Although the mechanism of the antioxidant effect is not fully understood, and the mechanism of its effect as vitamin E is not understood at all, the suggestion as to some relationship of those two effects is almost inescapable. The vitamin effect may be closely related to the antioxidant effect, except of course for the fact that the more specific effect of the vitamin requires a special structure in addition to the general feature of being a substituted hydroquinone. It may be left undecided whether the specific structure is just to make it more fat-soluble or to adapt it to any function as a coenzyme to some enzyme.

Hydroquinone is an efficient antioxidant¹. Although the mechanism of its action is not known in every respect, it can scarcely be doubted that this effect is in some way connected with its reversible oxidizability. However, also phenols with only one (or at least one unsubstituted) hydroxyl group are antioxidants². Here no reversible oxidation comparable with that of hydroquinone can take place. The reversible oxidation of hydroquinone leads to quinone, by a bivalent oxidation passing through the intermediate stage of a semiquinone. For monophenols, no such bivalent reversible oxidation is imaginable. However, a reversible univalent oxidation to a free radical is imaginable both for hydroquinone and for mono-phenols\*\*, including tocopherol. Such a radical would be a rather unstable compound. Ordinary oxidizing agents may not be able to produce the semiquinone radicals to any readily recognizable extent; yet, if a free radical may be produced only to a slight extent, not recognizable directly, the high energy content of the radical would make it a powerful reactant; just as the free OH radical, although never existing to any directly recognizable extent in an aqueous solution, has been recognized as a powerful reagent in many chain reactions.

However, any speculation about such free radicals is all too vague unless there is more direct evidence for their existence. It is the purpose of this paper to produce such evidence. It is based on a method devised by G. N. Lewis<sup>3, 4</sup> and consists of the following procedure. The substance to be oxidized is dissolved in an organic solvent such as, at the temperature of liquid air, will freeze to a homogeneous glass without crystallizing,

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<sup>\*\*</sup> At the present time, it will not be discussed whether even one unsubstituted hydroxyl group is necessary at all for the establishment of a free radical of comparable structure.

and is irradiated with ultraviolet light through quartz windows in a Dewar vessel. Such an irradiation may have two effects: one is, to raise the energy of some electron to a



Fig. I shows the absorption spectrum of irradiated a-tocopherol at .iquid air temperature, photographed with a spectrograph.

higher level. The spontaneous return of this electron to its ground level will be manifested by some luminescence, either fluorescence or phosphorescence of longer duration, according to conditions discussed by Lewis. In the second place, if there be an electron of sufficiently low ionization potential, the electron may be knocked out altogether, a process comparable to oxidation by a chemical oxidizing agent. At the temperature of liquid air and in the rigid medium molecular collisions are inhibited. Free radicals, once created, will accumulate to a concentration far above that permissible by thermodynamics, provided the electrons ejected are trapped in the molecules of the solvent and do not re-combine with the free radicals. In this case, no equilibrium in which the radical may be involved, can be established. Reactions such as dismutation, or dimeri-

zation of the radicals cannot occur. If the radical happens to be stable in so far as not to suffer a decay by a spontaneous unimolecular reaction (such as occurs in a radioactive atom), it will accumulate to a thermodynamically impermissible concentration. If the radical should be coloured, it could be seen in the frozen medium and remain as long as

the temperature is kept low. On slightly warming up the solution the colour should disappear. This may be taken as evidence for the that fact the colour belongs to a compound capable of existence to a noticeable extent only under conditions where the establishment of chemical equilibria is inhibited\*.

The colour produced in this way can, in suitable cases, be compared with the colour of free radicals produced by chemical oxidation. In fact, the absorption spectrum of the compound generated by either method was found to be identical<sup>3</sup> on working with such substances as asymmetrical dimethylp-phenylene diamine, or tritolylamine<sup>5, 6</sup>.

In this paper we shall describe the absorption spectra of several coloured substances considered as free semiquinone radicals prepared in this way from substances related

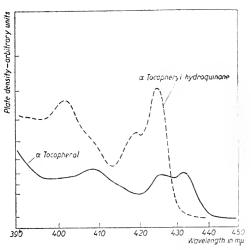


Fig. 2 shows tracings, obtained with a recording micophotometer, of the spectrum of irradiated a-tocopherol, and of irradiated a-tocopherylhydroquinone\*\*.

\*\* The authors are indebted to the Sun Chemical Company, New York, for their permission

to use their recording microphotometer.

<sup>\*</sup>According to Lewis and his associates, there may be still another effect: dissociation of a large molecule (such as tetraphenylhydrazin) either into two free radicals, or into a positive and a negative ion. Considering the structure of the compounds investigated, the possibility of such effects may be disregarded here. The fact that all the spectra obtained from the various compounds are similar, is further evidence as to the absence of any essential photodecomposition.

to tocopherol. They are all produced by irradiation of a solution in a mixture of ether, ethanol and pentane\*, in the volume proportions 5:2:5, respectively, with an ultraviolet lamp for the duration of a few minutes to about twenty minutes. Although the method is not suitable in its present form to tell anything about the yield, it may be stated, that the radical of tocopherol is produced with ease to a readily recognizable extent.

Among the substances irradiated during this experimental study there is, first of all, hydroquinone. It is irradiated, then the decay of the phosphorescence is awaited (usually several seconds), without lifting the vessel out of the liquid air environment. Now the colour in transmitted light is observed. It is yellow, its absorption spectrum consists of several bands in the visible, the maxima of which are reproduced in Fig. 3. The yellow substance is not quinone. Firstly, its absorption spectrum is different from

that of quinone; secondly, this colour vanishes on slightly warming up the frozen mixture. In addition, a spectrum of the same character is produced in this way from hydroquinone-monomethyl ether. This, of course, cannot be oxidized to the level of a regular quinone but there is no reason why it should not be oxidized to the level of a semiquinone.

Of the various tocopherols, samples of pure  $\alpha$ ,  $\delta$ , and  $\gamma$  tocopherol\*\* and several samples of commercially available  $\alpha$ -tocopherol were compared. The latter showed the same behaviour as the pure  $\alpha$ -tocopherol, whereas the  $\delta$  and  $\gamma$  compound showed, after irradiation, absorption bands slightly different from the  $\alpha$ -compound. Whereas the colour of the radicals from hydroquinone and its methyl-ether are yellow, that of all the tocopherols is red, of slightly orange tint.

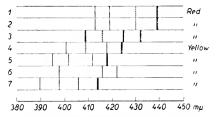


Fig. 3 shows the location of the absorption bands after irradiation as obtained according both to photographs such as Fig. 1 and to tracings such as Fig. 2.

- 1.  $\delta$ -tocopherol
- 2. γ-tocopherol
- 3. a-tocopherol
- 4. a-tocopheryl hydroquinone
- 5. hydroquinone monomethyl ether
- 6. durohydroquinone
- 7. hydroquinone

all the tocopherols is red, of slightly orange tint. This difference corresponds to the location of the absorption bands in Fig. 3.

The problem arises whether this "oxidation" by irradiation is a reversible one. Only in this case the substance could serve in metabolism as something analogous to a coenzyme of an oxidative enzyme. When tocopherol is chemically oxidized (say by ferric chloride), the first oxidation product obtainable is a quinone, tocopherylquinone<sup>7</sup>,

$$\begin{array}{c|c} CH_{3} \\ HO & CH_{2} \\ CH_{2} \\ H_{3}C & CH_{2} \\ C - C_{16}H_{33} \\ CH_{3} & CH_{3} \end{array}$$

a-tocopherol, parent substance of radical No. 3 in Fig. 3

$$\begin{array}{c|c} \operatorname{CH_3} & \operatorname{OH} \\ & \operatorname{CH_2} & \mid \\ \operatorname{CH_2} - \operatorname{C} - \operatorname{C}_{16} \operatorname{H}_{33} \\ & \mid \\ \operatorname{CH_3} & \operatorname{CH_3} \end{array}$$

a-tocopherylhydroquinone, parent substance of radical No. 4 in Fig. 3

\*\* We owe these to the courtesy of Distillation Products Corporation, Rochester, N.Y.

<sup>\*</sup> G. N. Lewis recommends isopentane. We had no trouble with ordinary commercial pentane. If the mixed solvent shows any inclination to crystallize at liquid air temperature, it can be corrected by adding slightly more ether.

which cannot be re-reduced directly to the original tocopherol because the phytol sidering is opened to make the quinone. When this quinone is reduced to its corresponding hydroquinone, and this "tocopherylhydroquinone" is irradiated under proper conditions, the absorption spectrum of the free radical is different from that produced by the irradiated tocopherol itself. It resembles, with its yellow colour, more that of the hydroquinone-methyl-ether. Hereby it is shown that the red radical produced from tocopherol does not involve the opening of the phytol side-ring. The preservation of the free radical will also be aided by the fact that the opening of the phytol ring represents a hydrolysis which cannot occur in the absence of water. There is, then, no reason, why the univalent oxidation of tocopherol, especially in a non-aqueous solvent, should not be reversible.

#### SUMMARY

Tocopherol, dissolved in a suitable mixture of organic solvents such as will, at the temperature of liquid air, form a homogeneous glass, is irradiated with ultraviolet light. A red colour is developed which disappears at slightly higher temperature. Similar observations are made with some other substances related to hydroquinones. The coloured substance is interpreted as a free semiquinone radical. Its possible function for the vitamine and the antioxidant effect of tocopherol is discussed.

#### RÉSUMÉ

Le tocophérol, dissous dans un mélange approprié de solvants organiques, mélange qui, à la température de l'air liquide, forme un verre homogène, est irradié au moyen de lumière ultraviolette. Une coloration rouge apparaît, qui redisparaît lorsqu'on élève quelque peu la température. Des observations similaires ont été faites avec quelques autres substances de nature hydroquinonique. La substance colorée est considérée comme étant un radical semiquinonique libre. Son rôle possible dans l'action vitaminique et antioxydante du tocophérol est discuté.

#### ZUSAMMENFASSUNG

Tocopherol, gelöst in einer geeigneten Mischung von organischen Lösungsmitteln, welche bei der Temperatur der flüssigen Luft zu einem homogenen Glas erstarren, wird mit ultraviolettem Licht bestrahlt. Es entsteht eine rote Färbung, welche bei höherer Temperatur wieder verschwindet. Ähnliches wird mit anderen Hydrochinon-ähnlichen Verbindungen beobachtet. Die gefärbte Substanz wird als ein Semichinon gedeutet und ihre mögliche Funktion bei der Rolle des Tocopherols als Vitamin und als Antioxidant erörtert.

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## THE COMBINATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE WITH GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

by

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It has been shown in a previous paper¹ that glyceraldehyde phosphate dehydrogenase from rabbit muscle contains one mole of diphosphopyridine nucleotide (DPN) per 50000 g of protein. This ratio did not change after prolonged dialysis against distilled water or after repeated recrystallizations from ammonium sulphate solutions. When an aqueous solution of the enzyme was treated with activated charcoal (norit) and filtered, DPN was removed. Addition of an excess of DPN and ammonium sulphate to the DPN-free enzyme solution resulted in the formation of crystals which contained the original ratio of DPN to protein. From these and other observations it was concluded that the enzyme contained firmly bound DPN. The fact that DPN could be removed with norit made it clear that the union between enzyme and coenzyme was not through a covalent bond.

Earlier work<sup>2</sup> had indicated that the dissociation constant of the enzyme with DPN, as estimated from the concentration of DPN at which the reaction with glyceraldehyde phosphate occurred at half maximal velocity, was of the order of 4·10<sup>-8</sup> M/ml. This agreed with a value obtained by Warburg and Christian<sup>3</sup> with yeast enzyme and free glyceraldehyde as substrate. According to existing criteria the constant so obtained is sufficiently large to permit easy separation of enzyme and coenzyme by dialysis or recrystallization. The fact that such a separation was not observed suggests either that the enzyme combines with DPN at two sites, one of which binds DPN more firmly than the other, or that the conclusions drawn from the kinetic measurements or from dialysis and recrystallization are not valid.

In the present paper experiments are described in which some aspects of the two-site hypothesis are tested. In order to make reactions of bound DPN measurable in a 1 cm cell at 340 m $\mu$  in the Beckman spectrophotometer, it is necessary to use enzyme concentrations of 2 to 4 mg per ml which are about 1000 times greater than those necessary to give good rates with added DPN and glyceraldehyde phosphate. Accordingly the reaction with glyceraldehyde phosphate is too rapid for convenient study, unless one works at a  $p_H$  far from the optimum. When glyceraldehyde is used as substrate, however, the reaction rate is conveniently measurable over a wide range of conditions, the slower reaction being due, as will be shown, to a low affinity of glyceraldehyde for the enzyme.

#### EXPERIMENTAL

The enzyme was prepared as previously described<sup>2</sup> and recrystallized four times. An aliquot of the crystal suspension in ammonium sulphate was centrifuged at about 10000 rpm, drained, and dissolved in 0.03 M sodium pyrophosphate — 0.003 M cysteine buffer at pH 8.3. This enzyme solution was prepared fresh for each experiment. The composition of reaction mixtures is given in the tables.

#### THE DISSOCIATION CONSTANT OF ENZYME AND BOUND DPN

The enzyme and bound DPN concentrations cannot be varied independently unless one resorts to partial removal of DPN with norit. The latter procedure introduces additional variables due to the instability of the DPN-free enzyme and so a dilution method was employed. It was possible to follow the reactions in the more dilute solutions by using cuvettes with a longer light path.

The experiment consisted in comparing the rates of reaction in two solutions identical in all concentrations except that of the enzyme-DPN complex. The results of such an experiment are described in Table I. It may be seen that the directly measured

TABLE I
THE DISSOCIATION OF ENZYME AND "BOUND" DPN

Two reaction mixtures were prepared, one with a total volume of 6 ml and the other of 30 ml. The former was in a cell of 2 cm and the latter in a cell of 10 cm length. Both reaction mixtures contained in moles per ml,  $6\cdot10^{-6}$  arsenate,  $3\cdot10^{-6}$  cysteine,  $5\cdot10^{-5}$  pyrophosphate (p<sub>H</sub> 8) and  $2\cdot10^{-6}$  DL-glyceraldehyde (the latter added to start the reaction). The two reaction mixtures differed however in that the 2 cm cell contained  $1.77\cdot10^{-8}$  and the 10 cm cell  $3.54\cdot10^{-9}$  M per ml of enzyme – DPN.

| 2 cm cell                                                                                         | 10 cm cell                                                  |  |  |
|---------------------------------------------------------------------------------------------------|-------------------------------------------------------------|--|--|
| $\begin{array}{ccc} \text{Cime} & & 2 \text{ cm cell} \\ \text{min}) & & \log  I_o/I \end{array}$ |                                                             |  |  |
| 0.022                                                                                             | 0.019                                                       |  |  |
| 0.037                                                                                             | 0.037                                                       |  |  |
| 0.053                                                                                             | 0.053                                                       |  |  |
| 0.067                                                                                             | 0.067                                                       |  |  |
| 0.079                                                                                             | 0.081                                                       |  |  |
| 0.091                                                                                             | 0.092                                                       |  |  |
| 0.099                                                                                             | 0.101                                                       |  |  |
| 0.223                                                                                             | 0.222                                                       |  |  |
|                                                                                                   | 0.022<br>0.037<br>0.053<br>0.067<br>0.079<br>0.091<br>0.099 |  |  |

<sup>\*</sup> After addition of glyceraldehyde phosphate.

rates were identical. This means that the decrease in rate due to the 5-fold dilution of enzyme-DPN complex was exactly compensated by the 5-fold increase in light path. Since the observed rate was proportional to the concentration of undissociated enzyme-DPN, it follows that no measurable increase in dissociation occurred on dilution. In order for this condition to hold, it would be necessary for the dissociation constant of enzyme-DPN to be of the order of 1·10<sup>-10</sup> M/ml or less. Since in fact no evidence of dissociation was obtained at all in this experiment, the above figure may be considered only to be an upper limit\*. An analogous dilution experiment with a small amount of enzyme and added DPN with glyceraldehyde phosphate as substrate showed a change

 $<sup>^{\</sup>star}$  In work which will be reported in detail at a later date it has been shown that bound DPN equilibrates rapidly with radioactive DPN labelled with  $P^{32}$ . This is in harmony with the conclusion that the bond between DPN and enzyme is not of the covalent type and that the bound DPN exhibits a finite dissociation.

in rate between DPN concentrations of  $4.4 \cdot 10^{-8}$  and  $4.4 \cdot 10^{-9}$  M/ml that is consistent with a dissociation constant of the order of  $4 \cdot 10^{-8}$  M/ml.

The fact that depending upon whether or not one measures bound DPN or added DPN, one gets apparent dissociation constants differing by a factor of *at least* 100 argues for the existence of two types of catalytic sites. We will designate the still hypothetical site with the higher DPN affinity as site I and the site with lower DPN affinity as site II and proceed to examine the conditions that would hold during the course of a reaction.

#### THE REACTION AT SITE I

In Table II is shown an experiment in which the reduction of bound DPN is studied as a function of glyceraldehyde concentration. The glyceraldehyde concentration in all

TABLE II

EFFECT OF CONCENTRATION OF GLYCERALDEHYDE

Reaction mixture consisted (in moles per ml) of 2.4·10<sup>-8</sup> enzyme – DPN, 6·10<sup>-6</sup> arsenate, 3·10<sup>-6</sup> cysteine, 5·10<sup>-5</sup> pyrophosphate (p<sub>H</sub> 8.3) and varying amounts of DL-glyceraldehyde.

| Time<br>in | 0.5 1                 | o-6  | 1.10                  | 6    | 2 · 10-6              |      |  |
|------------|-----------------------|------|-----------------------|------|-----------------------|------|--|
| min        | log I <sub>o</sub> /I | К*   | log I <sub>o</sub> /I | К*   | log I <sub>o</sub> /I | К*   |  |
| 1.5        | 0.028                 | 0.14 | 0.051                 | 0.27 | 0.087                 | 0.57 |  |
| 3.0        | 0.054                 | 0.15 | 0.088                 | 0.29 | 0.123                 | 0.55 |  |
| 4.5        | 0.072                 | 0.14 | 0.109                 | 0.29 | 0.140                 | 0.57 |  |
| 6.0        | 0.085                 | 0.14 | 0.123                 | 0.28 | 0.147                 | 0.57 |  |
| 7.5        | 0.096                 | 0.13 | 0.130                 | 0.26 | 0.150                 |      |  |
| 9.0        | 0.103                 | 0.13 | 0.136                 |      | 0.152                 |      |  |
| 10.5       | 0.108                 |      | 0.140                 |      | 0.152                 |      |  |
|            |                       | 0.14 | İ                     | 0.28 |                       | 0.56 |  |

<sup>\*</sup>  $K = 2.3/t \log A (A - x)$ , A = initial concentration of DPN.

cases was sufficiently higher than that of DPN so that it was virtually constant during the course of the reaction. Under these conditions the rate is described by a first order velocity constant. The fact that the first order constants increase linearly with initial glyceraldehyde concentration means that saturation of the enzyme with glyceraldehyde has not been approached. The dissociation constant of enzyme-glyceraldehyde is therefore very large.

At the concentrations of enzyme employed the amount of free DPN in equilibrium with the protein would be negligible if the dissociation constant at site I is less than I·10<sup>-10</sup>. The above reaction is therefore first order with respect to enzyme-DPN complex. This means that each enzyme molecule behaves as though it reacted only once.

When DPNH (in amounts equivalent to the bound DPN present) was added at the beginning of the reaction, it exerted an inhibitory effect. This is indirect evidence that DPNH as well as DPN is bound at site I. It is also possible to demonstrate in a direct manner that DPNH is bound. This was done by reducing the bound DPN in a solution containing 10 to 20 mg of enzyme per ml with excess glyceraldehyde phosphate and

arsenate and then precipitating the enzyme with ammonium sulphate at a final concentration of 85% saturation. It was found that 90% or more of the enzyme was precipitated and that the ratio of DPNH to protein in the precipitate was the same as that of DPN to protein in the original solution.

For the interpretation of reactions with added DPN an additional consideration is important, namely, whether added DPN can displace DPNH at site I. From the fact that DPN at site I is dissociable one would expect the same to hold for DPNH. The problem of displacement would then be resolved by a determination of the relative dissociation constants of enzyme with DPN and DPNH. Theoretically this could be done by determining the ratio of DPN to DPNH in the enzyme when enzyme-DPNH is precipitated in the presence of added DPN.

A preliminary experiment of this type is presented in Table III; it gives qualitative evidence that displacement of DPNH by DPN does occur and that the dissociation

### TABLE III COMPETITION BETWEEN DPN AND DPNH

DPN in enzyme was reduced by addition of arsenate and an equivalent amount of triosephosphate. Aliquots of the reduced enzyme were treated as follows. In (A) 0.5 ml of enzyme containing 12.5 mg of protein, + 0.1 ml of  $H_2O$ , was precipitated with 3 ml of saturated ammonium sulphate. In (B) 0.5 ml of enzyme + 0.1 ml of DPN solution (2.4·10<sup>-7</sup> M) was incubated for 3 minutes before being precipitated with ammonium sulphate. The precipitates were separated by centrifugation at 10000 rpm and dissolved in cysteine-pyrophosphate buffer.

|                                                                             |                    | A             |                         |                           |               | В             |                  |                           |  |
|-----------------------------------------------------------------------------|--------------------|---------------|-------------------------|---------------------------|---------------|---------------|------------------|---------------------------|--|
|                                                                             | Vol.<br>in ml      | Protein<br>mg | D <sub>340</sub>        | DPNH<br>M·10 <sup>7</sup> | Vol.<br>in ml | Protein<br>mg | D <sub>340</sub> | DPNH<br>M·10 <sup>7</sup> |  |
| Supernatant fluid Precipitate Pptate + HAsO <sub>4</sub> + triosephosph.*** | 3.6<br>3.1<br>3.25 | 1.3**         | 0.063<br>0.304<br>0.413 | 0.36<br>(1.49)<br>2.13    | 3.6<br>3.1    | 11.1**        | 0.275<br>0.175   | 1.57<br>o.86              |  |
|                                                                             |                    |               |                         | 2.49                      |               |               |                  | 2.43                      |  |

<sup>\*</sup> Calculated from optical density at 276 mµ.

\*\* Determined by biuret method.

constants of the oxidized and reduced forms with the enzyme are at least of the same order of magnitude. The chief objection that might be raised is that the high concentration of ammonium sulphate may change the equilibrium.

An analysis of the experiment shows that although a stoichiometric amount of glyceraldehyde phosphate was used, the reaction was only 70% complete when DPN was added. This value is calculated from the additional DPNH which appeared when excess triosephosphate and arsenate was added to the dissolved precipitate of the enzyme in experiment A. Accordingly there must have been residual triosephosphate in B when DPN was added. The excess DPN in B then drove the reaction to completion as shown by the DPNH recoveries in A and B.

Some of the DPNH in the supernatant fluid of B, therefore, arose by reduction of added DPN and hence did not represent DPNH displaced from the enzyme. A rough estimate of the amount actually displaced is (by comparison with experiments A) equal

<sup>\*\*\*</sup> An excess of glyceraldehyde phosphate was added in order to reduce DPN completely.

to the total amount in the supernatant of B (1.57 · 10<sup>-7</sup> M) minus the amount arising from residual triosephosphate,  $[(2.13 - 1.49) \cdot 10^{-7} = 0.64 \cdot 10^{-7}]$ , minus unprecipitated protein-DPNH (0.36 · 10<sup>-7</sup>). The net displaced DPNH is (1.57 — 0.64 — 0.36) · 10<sup>-7</sup> = 0.57 · 10<sup>-7</sup> M. A similar value is arrived at by comparing DPNH in the precipitated protein in A and B, namely (1.49 — 0.86) · 10<sup>-7</sup> = 0.63 · 10<sup>-7</sup> M.

#### THE REACTION AT SITE II

When reactions are studied with added DPN, site I is saturated, even at low enzyme concentrations and site II is saturated to an extent which depends upon its dissociation constant and the concentration of free DPN. Reaction will be expected to occur at both sites but the DPNH formed at site I will be displaced by DPN in solution and site I as well as site II will now have a "turnover". The reactions at both sites will be first order provided that at each site the affinity for DPN is the same as that for DPNH. Experimentally it was found that the rate remained first order when DPN was added, Table IV.

TABLE IV EFFECT OF ADDED DPN ON RATE OF REACTION

The enzyme concentration corresponded to  $3.4\cdot 10^{-8}$  M of bound DPN per ml, the  $p_H$  was 8.3 and the temperature 26°. No DPN was added in A, while in B and C, 3.4 and  $7\cdot 10^{-8}$  M per ml respectively was added, giving the total of concentrations of DPN shown in the table headings. The reaction was started by the addition of glyceraldehyde (final concentration as the D-form  $1.1\cdot 10^{-6}$  M per ml).  $K = 2.3/t \log A (A - x)$ , A being the initial concentration of DPN.  $V_0$  (initial velocity) = K times the initial concentration of DPN.

| Time | A                     | 1      | H            | 3      | C<br>10.4·10 <sup>-8</sup> M/ml |       |  |
|------|-----------------------|--------|--------------|--------|---------------------------------|-------|--|
| in   | 3.4.10-               | 3 M/ml | 6.8-10-      | 3 M/ml |                                 |       |  |
| min  | log I <sub>o</sub> /I | K      | $\log I_o/I$ | К      | log I <sub>o</sub> /I           | K     |  |
| 1.5  | 0.066                 | 0.25   | 0.105        | 0.19   | 0.112                           | 0.12  |  |
| 3.0  | 0.111                 | 0.24   | 0.191        | 0.20   | 0.217                           | 0.13  |  |
| 4.5  | 0.142                 | 0.24   | 0.248        | 0.19   | 0.302                           | 0.14  |  |
| 6.0  | 0.160                 | 0.23   | 0.290        | 0.19   | 0.371                           | 0.14  |  |
| 7.5  | 0.172                 | 0.22   | 0.320        | 0.18   | 0.426                           | 0.14  |  |
| 9.0  | 0.183                 | 0,22   | 0.343        | 0.18   | 0.468                           | 0.14  |  |
| ∞*   | 0.214                 | _      | 0.428        |        | 0.658                           |       |  |
|      |                       | 0.233  |              | 0.188  |                                 | 0.136 |  |
| Vo   |                       | 0.79   |              | 1.28   |                                 | 1.41  |  |

<sup>\*</sup> After addition of glyceraldehyde phosphate.

By multiplying first order velocity constants, K, by the initial concentrations of DPN one gets the initial velocity of the reaction,  $V_o$ , in terms of M. min<sup>-1</sup> ml<sup>-1</sup>. The observed increase in initial rate on addition of DPN can be seen to be approaching a maximum value which would correspond to the saturation of both sites with DPN. Because of the high concentration of enzyme, one cannot calculate the enzyme-coenzyme dissociation constants by the usual methods (which are based on the assumption that the concentration of free DPN is not appreciably diminished by combination with the enzyme). It is furthermore not possible from this experiment to reach unambiguous conclusions with respect to the number and type of catalytic sites.

#### EQUILIBRIUM CONSTANTS

The equilibria of reactions with free DPN and substrate using catalytic amounts of enzyme and of reactions between bound DPN and substrate with the protein present in quantities equivalent to the DPN may be formulated respectively as follows:

- a) DPN + 3-glyceraldehyde phosphate + HPO<sub>4</sub> =  $\frac{\text{enzyme} \setminus}{\text{OPNH}}$  DPNH + H+ + 1,3-diphosphoglyceric acid
- b) DPN-enzyme + 3-glyceraldehyde phosphate +  $HPO_4^{=}$   $\Longrightarrow$  DPNH-enzyme +  $H^+$  + 1.3-diphosphoglyceric acid.

In the former case which is a true catalytic reaction, the enzyme forms transient intermediates with a minute fraction of the substrate at any given time. Case (b) is in effect a different reaction in which not free DPN and DPNH but the corresponding protein complexes are reactants.

MEYERHOF AND OESPER<sup>4</sup> have carried out a detailed study of the reaction as represented by (a). Since one hydrogen ion enters the equilibrium, the equilibrium constant showed a dependence upon  $p_H$ . Equilibrium measurements were made with added DPN under conditions similar to those employed by MEYERHOF AND OESPER. About 30  $\gamma$  of enzyme per ml were used so that equilibrium was reached within one minute after addition of glyceraldehyde phosphate, even at low  $p_H$  values. Concentrations of DPN and glyceraldehyde phosphate in the stock solutions were determined optically by enzymatic methods.  $p_H$  was measured with a glass electrode in the reaction mixture at the end of the experiment. The values found for the equilibrium constants fall well within the range reported by MEYERHOF AND OESPER, Table V.

 $\label{eq:table_v} TABLE\ V$  equilibrium of reaction at different  $p_H$ 

The equilibrium is compared for catalytic amounts of enzyme (C) plus added DPN, and large amounts of enzyme (L) containing bound DPN. The initial and final concentrations are given in moles per liter. GAP = glyceraldehyde phosphate.

| Amount of   | Initial Concentrations |                      |                      | Final Concentrations |                      |                      | Present data         |                      | Data of M. and O.4 |              |
|-------------|------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|--------------------|--------------|
| enzyme      | DPN                    | GAP                  | PO <sub>4</sub>      | DPN                  | GAP                  | PO <sub>4</sub>      | K                    | РН                   | K                  | Рн           |
|             | · 105                  | · 103                | .103                 | .105                 | $\cdot$ 103          | .103                 |                      |                      |                    |              |
| L<br>C<br>L | 6.23<br>7.48<br>5.46   | 1.43<br>1.42<br>1.43 | 82.8<br>82.8<br>8.66 | 4.08<br>4.62<br>4.45 | 1.39<br>1.37<br>1.38 | 82.8<br>82.8<br>8.62 | 0.67<br>0.65<br>16.4 | 7.09<br>7.08<br>7.85 | 0.6–1.4<br>19.8*   | 7.15<br>7.85 |
| С           | 7.53                   | 1.43                 | 8.66                 | 6.35                 | 1.36                 | 8.60                 | 28.9                 | 8.10                 | 21-28              | 8.20         |

<sup>\*</sup> Calculated from Meyerhof and Oesper's data by means of their complete equilibrium equation.

For equilibrium measurements under the conditions of case (b) two parallel reaction mixtures were prepared which differed only in that one contained phosphate and the other arsenate. The former was used for equilibrium determination while the latter served for determination of the amount of DPN present in the enzyme. The value of the equilibrium constants that were obtained agree within experimental limits with those found with small amounts of enzyme and added DPN.

Although one cannot derive from these measurements evidence for the existence References p. 169.

of two catalytic sites, the following considerations are of interest. In case (a) the enzyme cannot contribute to the net free energy change which is fixed by the initial and final states of the free reactants. In case (b) two of the reactants have been altered by complex formation and the initial and final energy states are not the same as in case (a). However, since only the *difference* in initial and final states determines the net free energy change, case (b) may or may not have the same equilibrium constant as case (a). These considerations apply irrespective of the physical nature of the bonding forces involved and the number and type of binding sites.

It may be inferred from the kinetics that the protein has the same affinity for DPN as for DPNH\*. Conclusions concerning the relative dissociation constants of enzyme-DPN and enzyme-DPNH may also be drawn from a comparison of the equilibrium constants in (a) and (b). If the binding of the other reactants does not alter their energy differences then, from the equality of equilibrium constants, it follows that the dissociation constants of enzyme-DPN and enzyme-DPNH are equal.

#### PH OPTIMUM

The rate of the reaction of glyceraldehyde with enzyme DPN was measured at  $p_H$  8.4, 7.5, and 6.4 in cysteine-pyrophosphate buffer. The relative rates calculated from the first order velocity constants were as 100:30:9. This agrees with the  $p_H$  activity curve as determined previously with small amounts of enzyme (6  $\gamma$ /ml) and addition of DPN and glyceraldehyde phosphate as substrate<sup>2</sup>.

#### REACTION WITH LACTIC DEHYDROGENASE

It has been shown in a previous report¹ that enzyme DPN, after reduction by glyceraldehyde phosphate, was reoxidized by addition of sodium pyruvate and a purified preparation of lactic dehydrogenase from rabbit muscle. The simplest explanation of this result is that the bound DPNH has a small but finite tendency to dissociate and that it is the dissociated DPNH which reacts with the pyruvate-lactic dehydrogenase system. In these experiments lactic dehydrogenase was present in considerable excess, so that the rate of the reaction could not be measured.

The dissociation constant for lactic dehydrogenase and DPNH has been determined by Kubowitz and Ott5 who report a value of  $5 \cdot 10^{-9}$  M/ml. In experiment A, Table VI, the initial concentration of bound DPNH was  $\frac{2 \cdot 3 \cdot 0 \cdot 146}{1 \cdot 45 \cdot 10^7} = 2 \cdot 3 \cdot 10^{-8}$  M/ml. If the DPNH-enzyme dissociation constant were  $1 \cdot 10^{-10}$  M/ml, there would not be enough free DPNH in solution to give 25% saturation of lactic dehydrogenase and the rate of reaction would be much slower than in experiment C, where the concentration of added DPNH was  $3 \cdot 1 \cdot 10^{-8}$ M/ml or enough to saturate the enzyme. The fact that such a difference\*\*\*

<sup>\*</sup> This inference arises from the fact that in the presence of a large excess of glyceraldehyde and arsenate the reduction of bound and of added DPN may be described by a first order velocity constant. If one assumes that DPNH has the same affinity for the catalytic site as does DPN, then the first order kinetics may be shown to be due to the formation of DPNH which acts as a competitive inhibitor<sup>3</sup>.

<sup>\*\*</sup> Actually the rate was faster in A than in C. One possible explanation was that lactic dehydrogenase in C was acting in the absence of "protective" protein. In order to compensate for this difference, lactic dehydrogenase was added in other experiments to a solution containing the same amount of triosephosphate dehydrogenase the DPN of which had not been reduced. The rate of reaction of lactic dehydrogenase with "bound" and with added DPNH was then approximately the same.

#### TABLE VI

## REACTION OF "BOUND" DPNH WITH LACTIC DEHYDROGENASE SYSTEM

The DPN in 24 mg of glyceraldehyde phosphate dehydrogenase was first reduced by addition of glyceraldehyde phosphate and arsenate. One aliquot (A) was precipitated directly with ammonium sulphate, while another aliquot (B) was first exposed to 0.024 M iodoacetate before being precipitated with ammonium sulphate. The precipitates were separated by centrifugation, dissolved in cysteine-pyrophosphate buffer,  $p_H$  8.3, and pyruvate (1·10<sup>-5</sup> M/ml) was added. The reaction was started by the addition of a catalytic amount of lactic dehydrogenase. To reaction mixture (C) free DPNH was added in place of glyceraldehyde phosphate dehydrogenase containing bound DPNH.

| Time<br>in min | A                     |       | I                     | 3     | С                     |       |  |
|----------------|-----------------------|-------|-----------------------|-------|-----------------------|-------|--|
|                | log I <sub>o</sub> /I | Δ     | log I <sub>o</sub> /I | Δ     | log I <sub>o</sub> /I | Δ     |  |
| O              | 0.146                 |       | 0.146                 |       | 0.195                 |       |  |
| 1              | 0.112                 | 0.034 | 0.098                 | 0.048 | 0.169                 | 0.026 |  |
| 2              | 0.084                 | 0.062 | 0.071                 | 0.075 | 0.153                 | 0.042 |  |
| 3              | 0.068                 | 0.078 | 0.050                 | 0.096 | 0.140                 | 0.055 |  |
| 4              | 0.050                 | 0.096 | 0.037                 | 0.109 | 0.127                 | 0,068 |  |
| 5              | 0.045                 | 0.101 | 0.029                 | 0.117 | 0.113                 | 0.082 |  |

in rate was not observed leaves one with two alternatives. Either DPNH is more highly dissociated than has been assumed or lactic dehydrogenase can react with bound DPNH. The latter alternative would involve collisions between protein molecules which, from a kinetic standpoint, is not incompatible with a rapid rate of reaction.

In a final experiment we tried to see whether the lactic dehydrogenase system could reduce the DPN bound to the enzyme. The reaction mixture contained 4 mg of glyceraldehyde phosphate dehydrogenase per ml as a source of DPN, sodium lactate, cyanide (to trap the pyruvate formed) and a catalytic amount of lactic dehydrogenase. The bound DPN was reduced at a good rate as soon as the lactic dehydrogenase was added. The considerations mentioned above when the reverse reaction was discussed apply here as well.

#### IODOACETATE

Iodoacetate (0.004 M) completely inhibited the reduction of enzyme DPN by substrate. An enzyme solution of about 8 mg of protein per ml was prepared with pyrophosphate buffer at  $p_H$  8.4 containing no cysteine. Five minutes at 25° was allowed for reaction with iodoacetate before glyceraldehyde was added. A suitable control without iodoacetate was run simultaneously. This was necessary because the enzyme loses activity quite rapidly in the absence of cysteine. No enzymatic activity could be detected in the presence of iodoacetate. Whether or not a differentiation of two catalytic sites is possible by means of addition of smaller concentrations of iodiacetate has not been tried.

In experiment B, Table V, iodoacetate was added after the DPN bound to the enzyme had been reduced. The object was to see whether the inactivation of the enzyme by iodoacetate would influence the rate of reaction of bound DPNH with the lactic dehydrogenase system. As shown in Table V no difference could be detected.

This paper is presented as a token of esteem for the numerous scientific contributions of Otto Meyerhof.

#### SUMMARY

The theory has been examined that glyceraldehyde phosphate dehydrogenase from rabbit muscle contains two catalytic sites, having dissociation constants with DPN which differ by a factor of 100 or more. The facts in favour of a very slightly dissociated site are that the enzyme retains on recrystallization or dialysis a stoichiometric amount of DPN. From observations made in kinetic measurements this DPN does not measurably dissociate on five fold dilution of the enzyme. Furthermore, evidence is presented that DPNH is also bound to the enzyme and that it can be displaced by added DPN to an extent which indicates relative affinities of the protein for the oxidized and reduced forms of at least the same order of magnitude. The fact that bound DPN can be removed from the enzyme by adsorption on charcoal and that it exchanges rapidly with DPN labelled with P32 allows the conclusion (a) that the binding is not of the covalent type and (b) that bound DPN has a measurable dissociation.

Other approaches to the problem did not reveal differences between the reaction with enzyme-DPN and the reaction with a catalytic amount of enzyme plus added DPN. In both cases, in the presence of an excess of substrate, the reaction was first order with respect to the total DPN concentration, and the  $p_H$  optimum was the same. The equilibrium constants with bound and with added DPN were also the same. Iodoacetate inhibited the reaction at the bound site. Kinetic studies involving simultaneous reaction of bound and added DPN showed that with increasing concentrations of the latter a saturation value was approached, but the data could not be resolved to give an unequivocal answer in terms of two catalytic sites.

Enzyme DPNH was shown to react rapidly with lactic dehydrogenase plus pyruvate, or in the reverse reaction, bound DPN was found to react with lactic dehydrogenase plus lactate. On the basis of the assumption that bound DPNH has a very low dissociation, the observed rate of reaction with lactic dehydrogenase would have to be attributed to collisions between protein molecules.

In the light of available evidence the hypothesis that glyceraldehyde phosphate dehydrogenase has two catalytic sites which differ in their affinity for DPN requires further examination.

## RÉSUMÉ

Un examen a été fait de la théorie selon laquelle la déshydrogénase de l'aldéhyde phosphoglycérique du muscle de lapin posséderait deux positions catalytiques dont les constantes de dissociation avec le DPN différeraient par un facteur de 100 ou davantage. Les faits en faveur d'une position où la dissociation est très faible sont que l'enzyme, lors de la recristallisation ou de la dialyse, retient une quantité stoéchiométrique de DPN. D'observations faites au cours de mesures cinétiques, il découle que ce DPN ne dissocie pas d'une façon appréciable lorsqu'on dilue l'enzyme au cinquième. En outre, des preuves sont apportées que le DPNH est lui aussi lié à l'enzyme et peut être déplacé de cette combinaison par l'addition de DPN, jusqu'à une limite qui indique que les affinités relatives de la protéine pour la forme oxydée et pour la forme réduite sont en tout cas du même ordre de grandeur. Le fait que le DPN lié peut être éliminé de l'enzyme par adsorption à du charbon actif, et qu'il s'établit un échange rapide avec du DPN marqué au P³², permet de conclure: a) que le mode de liaison n'est pas du type covalent et b) que le DPN possède une dissociation mesurable.

D'autres méthodes d'approche du problème posé n'ont pas révélé de différences entre la réaction de la combinaison enzyme-DPN et celle d'une quantité catalytique d'enzyme plus du DPN additionné. Dans les deux cas, en présence d'un excès de substratum, la réaction était du premier ordre par rapport à la concentration totale en DPN, et le p<sub>H</sub> optimum était le même. Les constantes d'équilibre avec du DPN lié ou additionné étaient également identiques. L'acide iodacétique inhibe la réaction au point de liaison. Des études cinétiques impliquant la réaction simultanée de DPN lié et de DPN additionné ont montré que lorsque les concentrations de ce dernier augmentent, on tend vers une valeur de saturation, mais il n'a pas été possible d'ordonner les résultats de façon à donner une réponse non équivoque à la question de l'existence de deux positions catalytiques.

Il a été montré que le DPNH lié réagit rapidement avec la déshydrogénase lactique plus pyruvate, ou, en sens inverse, le DPN lié avec la déshydrogénase lactique + lactate. Si l'on assume que le DPNH lié dissocie très faiblement, la vitesse observée de la réaction avec la déshydrogénase lactique devrait être attribuée à des collisions entre des molécules de protéine ou à la formation de complexes enzymatiques organisés. A la lumière des faits établis, l'hypothèse que la déshydrogénase de l'aldéhyde phospho-glycérique possède deux positions catalytiques différant par leur affinité pour le DPN demande de nouvelles études.

#### ZUSAMMENFASSUNG

Es wurde die Theorie untersucht, welche besagt dass Glycerinaldehydphosphat-Dehydrogenase aus Kaninchenmuskel zwei katalytische Stellen besitzt, deren Dissoziationskonstanten mit DPN um

mehr als das Hundertfache von einander abweichen. Die Tatsachen, die für eine sehr wenig dissoziierte Haftstelle sprechen, sind die, dass das Enzym beim Umkristallisieren oder bei der Dialyse eine stöchiometrische Menge DPN zurückhält. Aus Beobachtungen bei kinetischen Messungen geht hervor, dass dieses DPN bei fünffacher Verdünnung des Enzyms nicht messbar dissoziiert. Obendrein werden Belege dafür erbracht, dass auch DPNH an das Enzym gebunden ist, und aus dieser Verbindung durch zugesetztes DPN verdrängt werden kann bis zu einem Grade, welcher relative Affinitäten des Proteins zur oxydierten und zur reduzierten Form von mindestens gleicher Grössenordnung anzeigt. Die Tatsache, dass gebundenes DPN durch Adsorption an Kohle aus dem Enzym entfernt werden kann, und dass die Austauschreaktion mit DPN welches mit P³² markiert ist eine rasche ist, erlaubt den Schluss: a) dass die Bindung nicht covalenter Art ist und b) dass gebundenes DPN messbar dissoziiert.

Andere Angriffsarten auf das gestellte Problem zeigten keine Unterschiede auf zwischen der Reaktion mit Enzym-DPN und der Reaktion mit einer katalytischen Menge Enzym plus zugesetztem DPN. In beiden Fällen war, in Gegenwart eines Überschusses an Substrat, die Reaktion erster Ordnung mit Bezug auf die gesamte DPN-Konzentration und das pH-Optimum war das Gleiche. Die Gleichgewichtskonstante mit gebundenem und mit zugesetztem DPN war ebenfalls dieselbe. Jodacetat hinderte die Reaktion an der Bindungsstelle. Kinetische Untersuchungen, bei welchen gleichzeitig gebundenes und zugesetztes DPN reagierte, zeigten an, dass man sich mit wachsender Konzentration des Letzteren einem Sättigungswert näherte; jedoch konnten die Ergebnisse nicht so dargestellt werden, dass sie eine unzweideutige Antwort auf die Frage gegeben hätten, ob zwei katalytische Stellen bestehen.

Es wurde gezeigt, dass gebundenes DPNH rasch mit Milchsäure-Dehydrogenase plus Pyruvat reagierte, oder in umgekehrter Richtung gebundenes DPN mit Milchsäure-Dehydrogenase plus Lactat. Auf der Grundlage der Annahme, dass gebundenes DPNH sehr wenig dissoziiert, müsste die beobachtete Reaktionsgeschwindigkeit mit Milchsäure-Dehydrogenase durch Zusammenstösse zwischen Proteinmolekeln erklärt werden, oder durch die Bildung von geordneten Enzym-Komplexen. Im Lichte der vorhandenen Belege gesehen bedarf die Hypothese, dass Glycerinaldehydphosphat-Dehydrogenase zwei katalytische Stellen besitzt, welche sich in ihrer Affinität für DPN unterscheiden, weiterer Untersuchung.

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## GÄRUNG UND PHYTOCHEMISCHE REDUKTION

von

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Rein chemische Erfahrungen haben vor Decennien den Gedanken nahe gelegt, dass der Abbau der Hexosen in der Natur über Stoffe der 3-Kohlenstoffreihe erfolge. Es ist namentlich die 1871 von Hoppe-Seyler aufgefundene Entstehung von d,l-Milchsäure aus Traubenzucker<sup>1</sup> gewesen, die auf diesen Gedanken geführt hat. Er wurde befestigt durch bestätigende und erweiternde Beobachtungen<sup>2</sup> von Schützenberger (1876), NENCKI UND SIEBER (1881) und KILIANI (1882). Eine Umwandlung etwa von d-Glucose zu einer der optisch aktiven\* Raumformen der Milchsäure oder auch nur eine einfache Depolymerisation zu Triosen, zu optisch aktivem Glycerinaldehyd oder zu Dioxyaceton, war nicht ausgeführt. Der umgekehrte Vorgang, die Condensation von Triosen zu racemischen Hexosen, war in den Jahren 1887-1890 verwirklicht; er bildet eine der Grundlagen der Zuckersynthesen von Emil Fischer. In frühe Zeit (1904) fallen die ersten physiologischen Versuche mit Glycerose, dem Gemisch von Dioxyaceton und d,l-Glycerinaldehyd, das schon damals4 in ziemlich reinem Zustande erhältlich war. Mit diesem Material haben Neuberg und Blumenthal<sup>5</sup> den ersten experimentellen Beweis dafür geliefert, dass Triosen im Tierkörper zu optisch aktiven Hexosen condensiert werden, und Glycogenbildner sind. Diese Feststellung ist dann vielfach bestätigt worden, so von Mostowsky, Parnas, Embden und Mitarbeitern, Ringer und Frankel, Stöhr<sup>6</sup> u.a. Diese und eine Reihe ähnlicher Befunde, d.h. Biosynthesen von Hexosen mittels niederer Zucker, waren als Beispiele einer Aldolcondensation verständlich. Der stereochemische gerichtete Verlauf war mit den Prinzipien der asymmetrischen Synthese erklärlich. Dagegen war der Mechanismus des biochemischen Zuckerabbaus unerforscht. Es fehlten z.B. alle Grundlagen für die Herleitung der Methylgruppe, wie sie für die typischen Produkte der Glycolyse, für Milchsäure und Weingeist, charakteristisch ist. Dieses Problem ist der Lösung zugeführt mit der 1911 begründeten Lehre von der Rolle der Brenztraubensäure für den Umsatz der Zucker. Damit war die Aera eingeleitet, in der die biochemische Zerreissung\*\* der 6-Kohlenstoffkette, die Desmolyse der Zymohexosen, zu Substanzen der 3-Kohlenstoffreihe experimentell bewiesen wurde. Mit der hälftigen Aufteilung der Hexose in 2 Mol Methylglyoxal-hydrat ( $C_6H_{12}O_6 = 2C_3H_6O_3$ ), die 1928-1929 Neuberg und Kobel<sup>8</sup> mit verschiedenen Enzympräparaten pflanzlicher und tierischer Provenienz herbeiführten, schien das Problem gelöst. Meyerhof und Loh-MANN<sup>9</sup> zeigten 1934, dass unmittelbare Vorläufer des isolierten Methylglyoxals die

\*\* Für den Vorgang der enzymatischen Trennung von -C-C- Bindungen hat sich die 1925 von Neuberg und Oppenheimer? eingeführte Bezeichnung Desmolyse eingebürgert.

<sup>\*</sup> Die Behauptung Duclaux's, dass im Sonnenlicht aus einer alkalischen Glucoselösung d-Milchsäure in grosser Ausbeute entstehe, ist nach Jacobsohn<sup>3</sup> auf eine Verwechslung mit optisch aktiven Saccharinsäuren zurückzuführen.

Triosen sind, und zwar in Form ihrer unter den Versuchsbedingungen zum Zerfall in Methylglyoxal neigenden Phosphorsäureester<sup>10</sup>. Das Ausgangsmaterial für das desmolytisch gebildete Methylglyoxal ist das Fructosediphosphat gewesen. Schon diese Tatsache sprach für eine hierbei massgebliche Rolle der Phosphorylierung. Eine solche war niemals abgelehnt. Es ist jedoch in manchen Darstellungen nicht beachtet, dass in den damaligen Formulierungen die Beteiligung der Phosphorsäure der Vereinfachung wegen fortgelassen wurde und ausdrücklich bemerkt ist, dass phosphorylierte Zwischenstufen und die Triosen dem gegebenen Abbild ungezwungen eingefügt werden können, sobald sie nachgewiesen sein würden<sup>11</sup>.

Gleichfalls in die 3-Kohlenstoffreihe führten drei andere biochemische Reaktionen der Zymohexosen: die 1917 bekannt gegebene Spaltung in Glycerin, Kohlendioxyd und Acetaldehyd  $C_6H_{12}O_6 = CH_2OH.CHOH.CH_2OH + CO_2 + CH_3.CHO$  (II. Vergärungsform), die 1919 beschriebene Zerlegung in Glycerin, Kohlendioxyd, Aethanol und Essigsäure  ${}_{2}C_{6}H_{12}O_{6} + H_{2}O = {}_{2}CH_{2}OH.CHOH.CH_{2}OH + {}_{2}CO_{2} + C_{2}H_{5}OH + CH_{3}.COOH$ (III. Vergärungsform), und schliesslich die Aufteilung der Hexose in aequimolekulare Mengen Glycerin und Brenztraubensäure C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> = CH<sub>2</sub>OH.CHOH.CH<sub>2</sub>OH + + CH<sub>2</sub>.CO.COOH (IV. Vergärungsform). Die letzte ist experimentell am spätesten (1929) begründet. Infolge der Ausschaltung des Carboxylase-Systems findet man hier Primärprodukte. Unter den Bedingungen des Abfangverfahrens ist dagegen noch carboxylatische Spaltung der Brenztraubensäure möglich, und es entstehen die Erzeugnisse der 2. Vergärungsform, während bei schwach alkalischer Reaktion, welche die biochemische Dismutation\* des Acetaldehyds begünstigt, die Stoffe der 3. Vergärungsform auftreten. Meyerhof, Lohmann und Kiessling<sup>12</sup> haben gelehrt, dass Glycerin wie Brenztraubensäure phosphorylierte Vorstufen haben, l-Glycerin- 1-phosphorsäure einerseits, Enol-phosphobrenztraubensäure, bzw. in Position 2 und 3 phosphorylierte d-Glycerinsäure anderseits. Phosphoglycerinsäure, die schon 1928 synthetisiert war<sup>13</sup>, ist 1930 in einer denkwürdigen Arbeit Nilsson's<sup>14</sup> als Produkt einer von Fluorid beeinflussten Zuckerspaltung durch Hefe entdeckt worden. Die ursprünglich schwer erhältliche Substanz konnten Neuberg und Kobel<sup>15</sup> mit biochemischer Methodik als schön kristallisierendes saures Bariumsalz leicht zugänglich machen. Sie zogen, wie auch NILSSON<sup>16</sup>, die Schlussfolgerung, dass die Verbindung als normales Zwischenprodukt der Glycolyse fungieren möge, da sie diese Substanz mittels Hefen und Milchsäurebakterien in Brenztraubensäure überführen und im Gegensatz zu freier Glycerinsäure\*\* vergären konnten. In Würdigung ihrer sich immer mehr offenbarenden Bedeutung ist sie von MEYERHOF UND EMBDEN als Glied in die Kette der obligatorischen Zwischenprodukte der Desmolyse eingereiht worden. Substrate der Carboxylase und Ketonaldehydmutase sind Brenztraubensäure und Methylglyoxal, und die später nachgewiesenen umbauenden Enzyme Isomerase, Phosphoglyceromutase, Enolase u.s.w. greifen ebenfalls an 3-Kohlenstoffverbindungen an. Somit ist es selbstverständlich, dass diese Substanzen in jedem

<sup>\*</sup> Wenn Hefe Acetaldehyd statt zur Dismutation zu carboligatischer Erzeugung von Acyloin verwendet, so ist nach L. Elion (*Biochem. Z.*, 169 (1926) 471) auch unter diesen Bedingungen, wie bei der 2., 3. und 4. Vergärungsform, Glycerin das Reduktionsaequivalent zur Oxydationsstufe Acetaldehyd.

<sup>\*\*</sup> Freie Glycerinsäure wird unter keiner Bedingung von Hefe vergoren. Das ist schon von C. Neuberg und J. Kerb (Ber., 47 (1914) 1308) und unter kritischer Berücksichtigung der Literatur später wieder von O. v. Schönebeck (Biochem. Z., 276 (1935) 421) dargetan. Dagegen greifen Bakterien, die Hefe evtl. verunreinigen, nach C. Antoniani (Biochem. Z., 267 (1933) 380) freie Glycerinsäure an. Siehe auch A. I. Virtanen, (Biochem. Z., 279 (1935) 262) und I. Tikka (Biochem. Z., 279 (1935) 264).

Schema der glycolytischen Processe zentrale Plätze einnehmen. Das Kernstück bleibt immer die primäre Desmolyse zur Stufe der 3-Kohlenstoffverbindungen. Das kommt in dem Schema von Neuberg<sup>17</sup> zum Ausdruck, das die bis zum Jahre 1933 festgestellten Tatsachen zu erklären versucht, und in den fortentwickelten Paradigmen von EMBDEN, DEUTICKE UND KRAFT<sup>18</sup>, sowie von MEYERHOF<sup>19</sup> und CORI<sup>20</sup>, wo die vor der eigentlichen Desmolyse liegenden Umformungen und die generelle Rolle der Phosphorylierung und Dephosphorylierung ausführlich berücksichtigt sind. Erhebliche Fortschritte sind zu verzeichnen, namentlich ist die Beteiligung der Cofermente und anorganischen Ergänzungsstoffe, sowie die Reversibilität der meisten Reaktionsfolgen erkannt. Was die primäre Desmolyse als den charakteristischen Ausdruck der Glycolyse anbelangt, so ist der Übergang der Hexosen zur Wertigkeitsstufe der Triosen die integrierende Reaktion geblieben. Auch die Massnahmen, die zur Abfangung, Anhäufung und Isolierung von Intermediärgebilden oder zu Stabilisierungsprodukten (Essigsäure, Glycerin) führen, sind prinzipiell von gleicher Art. Durch künstliche Eingriffe wird irgendwie die normale Korrelation der Biokatalysatoren gestört und die Weiterverarbeitung unterbunden, mag dies durch Fixierung eines Zwischenproduktes, Abschwächung eines der Partialagentien, durch Zusätze oder Verdünnung\*, durch Ferment-oder Coferment- ausschaltung oder spezielle Begünstigung einer der Enzymreaktionen ge-

Wir sind über die biochemische Bildungsweise diverser 3-Kohlenstoffkörper unterrichtet. Ungeklärt ist bis heute, wie das Glycerin entsteht, das in kleinen Mengen bei der normalen alkoholischen Gärung auftritt. Die Herleitung aus den Triosen läge nahe, da die rein chemische Reduktion des Dioxyacetons<sup>22</sup> wie des Glycerinaldehyds<sup>23</sup> zum Glycerin keine Schwierigkeiten bietet. Schon bevor MEYERHOF die bedeutsame Isolierung der phosphorylierten Triosen in Substanz geglückt war, hat man mehrfach in Gärflüssigkeiten und Zellelementen kleine Mengen eines Materials beobachtet\*\* (IWANOFF, v. EULER, WARKANY, KLUYVER, STRUYK, BOYLAND, DISCHE u.a.), das bei der Destillation mit H<sub>2</sub>SO<sub>4</sub> von 20% das leicht nachweisbare Methylglyoxal liefert. Wahrscheinlich handelt es sich um gebundene, nicht um freie Triosen. Dass erstere durch Dismutationsreaktionen Glycerophosphat liefern können, haben Meyerhof und Kiessling<sup>24</sup> dargetan. Die erste in der Hefe aufgefundene Phospho-monoesterase ist die Glycerophosphatase. Sie spaltet, wie frühzeitig<sup>25</sup> dargetan ist, leicht die Salze der Glycerinphosphorsäure. So erscheint es möglich, dass die bei der 2. und 3. Vergärungsform gebildeten Stoffe, insbesondere das Glycerin, über phosphorylierte Vorstufen entstehen. Es wäre bei den jetzt erkannten Beziehungen zwischen enzymatischer Zuckerspaltung und Bioreduktion<sup>26</sup> auch denkbar, dass die Triosenphosphate zunächst der Dephosphorylierung anheimfallen und dann der phytochemischen Reduktion zu Glycerin unterliegen. Die nachstehend beschriebenen Versuche mit monomolekularem Dioxyaceton und d,l-Glycerinaldehyd lehren, dass keiner dieser Stoffe durch gärende Ober- und Unterhefe in Glycerin übergeführt wird. Da beide Triosen quantitativ übrigbleiben, scheidet auch die Eventualität aus, dass

\* Zur Theorie des Verdünnungseffekts, siehe F. Lynen<sup>21</sup>.

<sup>\*\*</sup> Lit. s. bei M. Kobel und C. Neuberg, 35. Meeting of the Soc. of American Bacteriologists, Philadelphia 1933; Biochem. Z., 269 (1934) 411 und 273 (1934) 445. Sie konnten durch zweckmässige Versuchsanordnung die bis dahin nur als minimal befundene Quantität < 1% auf 31% steigern. Hinzuzufügen ist als allem Anschein nach älteste einschlägige Angabe eine Notiz von F. Bordas und de Razkowski (Compt. rend., 126 (1898) 1050). Ihr zufolge sollen in umgeschlagenen (turned) französischen Weinen 3 Bakterienarten vorkommen, die Glucose spurenhaft in Dioxyaceton umwandeln. Experimentell ist diese Behauptung nicht hinreichend gestützt, vielleicht hat es sich um Acetylmethylcarbinol gehandelt.

eine Componente des racemischen Glycerinaldehyds in Reaktion träte. Im Gegensatz zu den Triosenphosphaten sind somit die freien Triosen für gewöhnliche Hefe (s. S. 174) unter den obwaltenden anaeroben Bedingungen keine angreifbaren Substrate. Dass Phosphorylierung die biologische Dignität einer Substanz völlig verändert, ist ausser an dem erwähnten Beispiel der Glycerinsäure (s. S. 172) auch sonst beobachtet, so von Pringsheim<sup>27</sup> bei d-Galactose-phosphat und namentlich von Warburg<sup>28</sup> und Dickens<sup>29</sup> für die Oxydasen der Glucose-6-phosphorsäure bezw. 6-Phosphogluconsäure. Die P-freien Stoffe sind keine Substrate für diese Enzyme.

Die Resistenz der Triosen beruht nicht auf einer Schädigung der benutzten Hefe durch die 3-Kohlenstoffzucker. Die abzentrifugierte Hefe erweist sich als ungeschwächt. In Gegenwart beider Triosen werden zugesetzte Zymohexosen glatt vergoren\*. Der von Lehmann und Needham³0 angegebene Einfluss des Glycerinaldehyds auf die glycolytischen Vorgänge macht sich nicht geltend, er ist auch in den Versuchen von Neuberg und Hofmann³1 nicht zu Tage getreten.

Das Verhalten der Triosen ist insofern unerwartet, als die nahestehenden Substanzen Milchsäurealdehyd, CH<sub>3</sub>.CHOH.CHO, und Acetol, CH<sub>3</sub>.CO.CH<sub>2</sub>OH, die als Desoxyderivate vom Glycerinaldehyd und Dioxyaceton aufgefasst werden können, und das Anhydrid der Triosen, das Methylglyoxal, CH<sub>2</sub>:CH(OH).CHO, der Bioreduktion zu dem mit Glycerol nahe verwandten Propylenglycol zugänglich sind<sup>23</sup>.

Die normale Funktion der benutzten Hefe offenbart sich ferner in Versuchen, die im Anschluss beschrieben seien, obzwar sie mit der Glycerinfrage als solcher nichts zu tun haben. Die phytochemische Reduktion des Cyclopentanons zum Cyclopentanol sowie die des d- und d,l-Campherchinons (2,3-Dioxycamphans) gelingt ohne Schwierigkeiten. Sie wird im letzten Falle halbseitig vollzogen, indem in der Hauptsache 3-Oxy-campher entsteht. Die Bioreduktion des d,l-Campherchinons verläuft partiell asymmetrisch. Dasselbe trifft für die phytochemische Reduktion des d,l-Methyl-n-propylacetaldehyds (Isocapronaldehyds) zu, die 2-Methyl-pentanol-1 mit einem Überschuss an linksdrehender Form liefert.

Auf Kosten vergärender Zucker ist somit die Bioreduktion in der Cyclopentanreihe und bei einem o-Chinon der hydroaromatischen Reihe möglich. Selbst ein so oberflächenaktiver Stoff wie der erwähnte Hexylalkohol verhindert den Eintritt der Bioreduktion nicht.

Der Beginn der hier mitgeteilten Versuche reicht länger zurück. Zu verschiedenen Zeiten haben daran mitgearbeitet Prof. Dr. N. N. Iwanoff, Leningrad, Dr. Hilda Lustig, New York, und Dr. Elisabeth Peiser, Berlin. Ihnen allen schulde ich Dank. Ich statte ihn in trauernder Erinnerung ab, alle drei weilen nicht mehr unter den Lebenden.

#### A. VERSUCHE MIT GLYCERINALDEHYD

Kristallisierter d,l-Glycerinaldehyd ist jetzt unschwer zugänglich<sup>32</sup>. Wird er in wässriger Lösung 24h bei Zimmertemperatur aufbewahrt, so vollzieht sich nach Wohl und Neuberg<sup>33</sup> der Übergang in die monomolekulare Form. Er wurde in 1.0, 0.5 und 0.25% Concentration verwendet.

In je 100 ml der Glycerinaldehydlösung wurden 10 g Rohrzucker oder Glucose gelöst. Auf Zugabe von 2–3 g obergäriger Brennereihefe trat bei 25° schnelle Gärung ein,

<sup>\*</sup> Glycerinaldehyd kann sogar als Aktivator der alkoholischen Zuckerspaltung fungieren: C. Neuberg und M. Ehrlich, *Biochem. Z.*, 101 (1920) 242.

die 2-3 Tage anhielt. Dann war alle Hexose verschwunden. Mit mehr Hefe wurde keine neue Gärung entfacht. Die schon in der Kälte eintretende Reduktion von Fehling'scher Mischung lehrte, dass unveränderte Triose vorhanden war.

Obgleich Methoden zur Bestimmung von Triose neben Hexose ausgearbeitet sind<sup>34</sup>, erübrigte sich deren Anwendung, da keine Hexosen mehr zugegen waren. Die zentrifugierten Flüssigkeiten, die kein Drehungsvermögen aufwiesen, zeigten gegen Fehling'sche Mischung dasselbe Reduktionsvermögen, wie die ursprüngliche Glycerinaldehydlösung; die Reduktionskraft der Triose ist schon von Wohl<sup>35</sup> ermittelt.

Verdoppelung der Mengen von Hexose und Hefe sowie erneuter Zusatz von Glucose und Hefe nach beendeter Gärung (in toto 3 Mal) änderte nichts an dem Ergebnis, so wenig wie die Heranziehung einer anderen Hefesorte (untergäriger Bierhefe). Eine phytochemische Reduktion des d,l-Glycerinaldehyds war nicht nachweisbar.

## B. VERSUCHE MIT DIOXYACETON

Die Versuche mit monomolekularem Dioxyaceton wurden wie die mit Glycerinaldehyd ausgeführt. Das Ergebnis war gleich, alle Ketotriose blieb unverändert.

Kristallisiertes Dioxyaceton ist nach Neuberg und Hofmann³6 in einfacher Weise erhältlich. Bei richtiger Arbeitsweise kristallisiert die Ketotriose direkt in einer Ausbeute von 77%, berechnet auf das in Arbeit genommene Glycerin, praktisch rein aus\*. Durch Aufarbeitung der Mutterlauge über das 2.4–Dinitrophenylhydrazon³² kann man noch 10–14% an kristallisiertem Dioxyaceton, in toto also 90%, gewinnen. Der Rest dürfte das von Levene und Walti³³ beschriebene polymere Condensationsprodukt enthalten.

Man kann sich von den mitgeteilten Tatsachen auch durch Anstellung der Versuche in kleinstem Umfange überzeugen. Statt eines titrimetrischen Verfahrens wählt man dann die Methode der Destillation mit  $\mathrm{H_2SO_4^{39}}$ . Es entsteht dabei quantitativ Methylglyoxal, und dieses kann jetzt in  $\gamma$ -Bereichen bestimmt werden<sup>40</sup>. Voraussetzung ist natürlich, dass keine Spezialhefen in Anwendung kommen, die Triosen angreifen, sei es durch Condensation zu Hexosederivaten<sup>41</sup>, sei es durch wirkliche Vergärung<sup>31</sup>.

## C. VERSUCHE MIT CYCLOPENTANON

Die Anstellung kann in der früher² für 2-Methylcyclohexanon angegebenen Weise geschehen. Zur Trennung von unverändertem Keton schaltet man zweckmässig eine Rektification über p-Nitrophenylhydrazin oder 2.4-Dinitropenylhydrazin ein. Das Cyclopentanol vom Siedepunkt 141° wurde in einer Ausbeute von 42% isoliert.

## D. VERSUCHE MIT d- UND d, l-CAMPHERCHINON

Die phytochemische Reduzierbarkeit der Diketone ist am Beispiel des Diacetyls aufgefunden<sup>26</sup>. Auch andere Polyketone sind der Hydrierung durch gärende Hefe zugänglich, solche der aliphatischen, aromatischen und heterocyclischen Reihe<sup>26</sup>. Im

<sup>\*</sup> Mit sehr ähnlicher Methodik haben auch Underkofler und Fulmer<sup>37</sup>a gute Resultate erzielt. Die von ihnen erhaltene Ausbeute ist etwas geringer gewesen. Die von ihnen angegebenen 80% beziehen sich nämlich nicht auf eingesetztes Glycerin, sondern auf Prozente von reduzierender Substanz. Diese besteht ausserdem nicht nur aus Triose, vielmehr ist nach Bousfield, Wright und Walker<sup>37</sup>b ein stärker reduzierender Körper beigemengt.

Campherchinon liegt ein bequem zugänglicher Vertreter von Diketonen der hydroaromatischen Reihe vor.

d-Campherchinon und d,l-Campherchinon werden von gärender Hefe unschwer und in erheblichem Ausmasse reduziert. Die Hydrierung konnte zum 2,3-Dioxycamphan führen, aber auch zu einem Oxy-oxo-campher. Das angewendete Campherchinon ging in einen Oxycampher über. Drehungen und Schmelzpunkte der Derivate liegen denen des 3-Oxy-camphers (2-Oxo-3-oxy-camphans) am nächsten. Die physikalischen Daten stimmten nicht genau damit überein, sondern sind ganz ähnlich wie bei dem Material, das durch Verfütterung von 2,3-Dioxocamphan an Hunde entsteht. Hier tritt neben 2-Oxy-3-oxo-campher ein nicht näher charakterisierter 3-Oxycampher auf<sup>42</sup>. Auch die rein chemische Reduktion des Campherchinons liefert ein Isomerengemisch<sup>43</sup>.

Auf alle Fälle findet eine partielle Bioreduktion statt. Sie ergreift nur eine der beiden Carbonylgruppen. Dass die phytochemische Reduktion in Stufen erfolgt, ist für die Umwandlungen des Diacetyls, des Furils und auch sonst nachgewiesen<sup>26</sup>. Oxyketone sind Zwischenglieder bei der Entstehung der Glycole. Beim Benzil ist bislang überhaupt nur die biochemische Bildung von Benzoin zu erzielen gewesen<sup>26</sup>. Ob unter den Bedingungen einer forcierten langanhaltenden phytochemischen Reduktion, die nach F. G. FISCHER auch Doppelbindungen erfasst<sup>26</sup>, die zweite Carbonylgruppe betroffen werden kann, bleibe dahingestellt.

Das d-Campherchinon wurde nach der Vorschrift von Evans, Ridgion und Simonsen<sup>44</sup> bereitet; aus Ligroin umkristallisiert schmolz es bei 198°.  $[a]_D=-92^\circ$ .

Eine Lösung von 10 g d-Campherchinon in 50 ml Alkohol lässt man zu dem gärenden Gemisch von 250 g Bäckerhefe und 2.5 Litern 10% Rohrzuckerlösung fliessen. Bei langsamem Zusatz wird die  $\mathrm{CO_2}$ -Entwicklung nicht unterbunden. Der Eintritt der Umwandlung ist ohne weiteres daran zu erkennen, dass die vom Chinon herrührende gelbe Farbe verschwindet. Nach 2-tägiger Digestion bei Zimmertemperatur saugt man die Hefe ab und schüttelt das Filtrat mit Aether aus. Nach Trocknen des Aetherextraktes über Natriumsulfat wurde das Lösungsmittel abdestilliert. Es hinterblieb ein farbloser kristallinischer Rückstand, der, aus Petroläther umkristallisiert, bei 200–202° schmolz. a in 11% iger alkoholischer Lösung im 1 dm – Rohr = + 3.83°.  $[a]_D = +$  34.9°. Ausbeute 6.3 g. Aus dem Hefeschlamm liess sich mit Wasserdampf nur eine ganz geringe Menge einer flüchtigen Substanz abtreiben, die vernachlässigt werden kann.

Zur Identifizierung wurde das Semicarbazon dargestellt.

Nach der Vorschrift von Bredt und Ahrens<sup>45</sup> wurden 0.42 g Semicarbazid-chlorhydrat und 0.35 g Kaliumacetat in Wasser gelöst, 0.5 g Substanz und soviel Methylalkohol hinzugegeben, dass eine klare Lösung entstand. Nach eintägigem Stehen schied sich ein Öl ab, das nach starker Abkühlung und Reiben mit einem Glasstabe kristallinisch erstarrte. Es wurde auf Ton abgepresst und aus Petroläther umkristallisiert. Die Verbindung schmolz bei 189°.  $\alpha$  in 6% alkoholischer Lösung im 1 dm – Rohr = + 0.26°  $[\alpha]_D = +$  4.4°.

$$C_{10}H_{16}O: N.NH.CO.NH_2.Ber.N = 18.7\%$$
; gef.  $N = 18.9\%$ 

8 g d,l-Campherchinon (aus synthetischem d,l-Campher bereitet) wurden mit 200 g Zucker und 200 g Hefe in 2 l Wasser vergoren. Das Filtrat wurde mit Aether extrahiert und der Aetherrückstand aus Petroläther umkristallisiert. Er schmolz bei 200–203°. Ausbeute 5 g an "3-Oxycampher".  $\alpha$  im 1 dm – Rohr in 10% alkoholischer Lösung = + 0.47°. [ $\alpha$ ]<sub>D</sub> = + 4.7°. Die phytochemische Reduktion verläuft also partiell asymmetrisch.

## E. VERSUCHE MIT ISOCAPRONALDEHYD (d, l-METHYL-n-PROPYL-ACETALDEHYD)

Die Arbeitsweise für die phytochemische Reduktion des verwendeten Isocapronaldehyds schloss sich an diejenige an, welche für die entsprechende Umwandlung des Isovaleraldehyds angegeben ist<sup>26</sup>.

10 g des racemischen Ausgangsmaterials (Kp 115–116°) lieferten 6.5 g 2-Methylpentanol-1 (Kp 147–149°). Dieser Hexylalkohol zeigte (unverdünnt) im 2 dm – Rohr eine Linksdrehung von  $\alpha=-0.9$ °. Für ein synthetisch gewonnenes Produkt, das vielleicht keine maximale Drehung besessen hat, ist in der Literatur<sup>46</sup> [a]<sub>D</sub> = — 1.25° angegeben.

#### ZUSAMMENFASSUNG

Im Anschluss an Betrachtungen über Entstehung, Verhalten und Bedeutung der 3-Kohlenstoffkörper, insbesondere der freien wie phosphorylierten Triosen, wird folgendes gezeigt: Gewöhnliche obergärige und untergärige Hefen, die Dioxyaccton und Glycerinaldehyd nicht vergären, bewirken keine phytochemische Reduktion der beiden Triosen zu Glycerin. Die 3-Kohlenstoffzucker werden nicht verändert. Sie sind in Konzentrationen von 1% für Hefe ungiftig und verhindern die glatte Vergärung zugefügter Zymohexosen nicht. Die Resistenz der Triosen gegen phytochemische Reduktion ist insofern bemerkenswert, als die Desoxytriosen, Acetol und Milchsäurealdehyd, ebenso wie das Anhydrid der Triosen, das Methylglyoxal, unter vergleichbaren Bedingungen zu dem mit Glycerol nahe verwandten Propylenglykol reduziert werden.

Die verwendeten Hefen sind zu Bioreduktionen durchaus geeignet befunden worden. Sie führen Cyclopentanon in Cyclopentanol, d- und d,l-Campherchinon durch Bioreduktion einer Carbonylgruppe in Oxycampher und Isocapronaldehyd in 2-Methylpentanol-1 über. Sobald dazu die Möglichkeit besteht, verläuft die phytochemische Reduktion asymmetrisch. Diese selber ist nunmehr auch in der Cyclopentanreihe und bei einem Diketon der hydroaromatischen Reihe verwirklicht worden.

#### SUMMARY

In connection with considerations about the origin, behaviour, and significance of  $C_3$ -substances, particularly free as well as phosphorylated trioses, it has been shown that: Ordinary top and bottom fermentation yeasts, which do not ferment dihydroxyacetone or glyceraldehyde, effect no phytochemical reduction of the two trioses to glycerol. The  $C_3$ -sugars are unchanged. They are not toxic to yeast in concentrations of 1%, nor do they inhibit the smooth fermentation of added zymohexoses. The resistance of the trioses to phytochemical reduction is noteworthy insofar as the desoxytrioses, monohydroxyacetone and lactic aldehyde, just like the triose anhydride, methylglyoxal, are reduced to propylene glycol (which is closely related to glycerol) under comparable conditions.

The yeasts used have been found to be entirely suitable for bioreductions. They convert cyclopentanone into cyclopentanol, d- and d,l-camphorquinone by bioreduction of a carbonyl group into hydroxycamphor, and isocaproic aldehyde into 2-methylpentanol-1. As soon as the possibility exists, the phytochemical reduction takes an asymmetric course. This has now been carried out in the

cyclopentane series and with a diketone of the hydroaromatic series.

#### RÉSUMÉ

A la suite de considérations sur la formation, le comportement et l'importance des corps à trois atomes de carbone, spécialement des trioses, tant libres que phosphorylées, on montre ce qui suit:

Des levures hautes ou basses ordinaires, qui ne font pas fermenter la dioxyacétone et l'aldéhyde glycérique, ne provoquent pas davantage de réduction phytochimique de ces deux trioses en glycérine. Les deux corps ne sont pas transformés. A la concentration de 1%, ils ne sont pas toxiques pour la levure et n'inhibent pas la fermentation régulière de zymohexoses additionnés. La résistance des trioses à la réduction phytochimique est d'antant plus remarquable que les désoxytrioses, l'acétol et l'aldéhyde lactique, de même que l'anhydride des trioses, le méthylglyoxal, sont réduits en propylèneglycol, proche parent de la glycérine, dans des conditions comparables.

Les levures utilisées ont été trouvées parfaitement aptes à effectuer des réductions biochimiques.

Elles transforment la cyclopentanone en cyclopentanol; la d- et la d,l-camphoquinone donnent, par réduction de l'un des deux groupes carbonyle, de l'oxycamphre; l'aldéhyde isocaproïque fournit le 2-méthylpentanol-1. Dès que la possibilité en est donnée, la phytoréduction prend un cours asymétrique. Cette phytoréduction a maintenant été réalisée aussi dans la série cyclopentanique et chez une dicétone de la série hydroaromatique.

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## ESSAIS DE BILANS DE LA FERMENTATION ALCOOLIQUE DUE AUX CELLULES DE LEVURES

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## I. LES DIVERS PRODUITS DE LA FERMENTATION ANAÉROBIE

## 1. Essai d'un bilan des produits secondaires de la fermentation

Très peu d'auteurs se sont attachés à établir un bilan complet de la fermentation alcoolique. Récemment, E. Peynaud, au laboratoire de Bordeaux, s'est attaché à suivre les divers produits formés par la levure vivante à côté de l'alcool; les premiers résultats relatifs à l'acide acétique ont paru déjà en 1939¹; les résultats principaux ont été publiés en 1946, 1947² et 1948. Ils vérifient une hypothèse sur l'origine des produits formés publiés par l'auteur de ces lignes dès 1936³. Le travail analytique considérable fourni par Peynaud n'a pas eu pour seul résultat d'accumuler des chiffres, ou même de vérifier des hypothèses; il a apporté quelques notions nouvelles plus ou moins inattendues.

L'hypothèse publiée en 1936<sup>3</sup> et vérifiée depuis, était la suivante: le glycérol prend naissance dans une fermentation glycéropyruvique:

I. 
$$C_6H_{12}O_6 = CH_2OH.CHOH.CH_2OH + CH_3COCO_2H$$

L'acide pyruvique formé est décarboxylé en acétaldéhyde; l'acétaldéhyde est dirigé vers 3 voies différentes:

2. a) elle est dismutée en alcool et acide acétique

$$_{2}$$
CH $_{3}$ CHO + H $_{2}$ O = CH $_{3}$ CH $_{2}$ OH + CH $_{3}$ CO $_{2}$ H

b) elle est condensée en acétylméthylcarbinol, réduit ensuite en 2–3 butylèneglycol

3. 
$$CH_3CHO + CH_3CHO + AH_2 = CH_3CHOH CHOH CH_3 + A$$

c) elle est condensée en acide succinique, avec formation corrélative de 3 molécules d'alcool

4. 
$$5CH_3CHO + 2H_2O = CO_2HCH_2CO_2H + 3CH_3CH_2OH$$

La voie a avait été décrite par C. Neuberg sous le nom de fermentation alcaline; la voie b avait été décrite par le même auteur pour levures fermentant en présence d'acétaldéhyde; la voie c n'avait à ma connaissance pas été envisagée. Pasteur avait déjà en 1861 affirmé que l'acide succinique ne pouvait provenir que du sucre, car la masse d'acide succinique formée pouvait atteindre 2 fois la masse de levure formée; Ehrlich avait fait en 1911 une autre hypothèse, qui a été depuis généralement admise sans preuve expérimentale sérieuse, à savoir que l'acide succinique proviendrait de Bibliographie p. 191/192.

l'acide glutamique. Malheureusement, les levures sont pauvres en acide glutamique, et la quantité d'acide succinique trouvée est 20 fois au moins la quantité d'acide glutamique de la levure qui l'a engendrée; les huiles de fusel apparaissent effectivement en quantités 10 à 20 fois plus faibles que l'acide succinique, ce que montrent par exemple les travaux de Claudon et Morin en 1887.

L'hypothèse de 1936 conduit à une équation que voici: entre le nombre g de molécules de glycérol, a d'acide acétique, b de butylèneglycol, m d'acétylmethylcarbinol, s d'acide succinique, h d'acétaldéhyde présentes côte à côte dans le milieu à un moment quelconque de la fermentation, doit exister la relation:

5. 
$$g = 2a + b + 2m + 5s + h$$
.

L'analyse de plus de 60 fermentations conduites dans des milieux divers, et avec diverses levures, a permis à E. Peynaud d'établir l'équation empirique<sup>4, 5, 7</sup>:

6. 
$$\sum = 2a + b + 2m + 5s + h = 0.9 g$$

ce qui signifie que 10% de l'acétaldéhyde donne des produits qui échappent pour le moment à l'analyse. Peynaud a trouvé qu'il se formait un peu d'acide citrique, ou du moins d'un acide en ayant tous les caractères analytiques (insolubilité du sel de baryum dans l'alcool à 30°, formation d'acétone par oxydation permanganique à l'ébullition à  $p_H$   $4^{10}$ , 11). Si l'on admet que l'acide citrique se forme suivant l'équation de bilan suivante (qui n'a nullement l'ambition de représenter la marche réelle de la formation de l'acide citrique):

7. 
$$9CH_3CHO + 4H_2O = CO_2H.CH_2COH CH_2 CO_2H$$
 
$$CO_2H + 6CH_3CH_2OH$$

l'introduction de l'acide citrique c avec le coefficient 9 dans l'équation 6 aboutit à un bilan se bouclant aux erreurs expérimentales près:

8. 
$$2a + b + 2m + 5s + h + 9c = g$$

## 2. Relation entre le CO2 et l'alcool

Des équations 2, 3, et 4, on peut déduire de même une relation entre le CO<sub>2</sub> dégagé au cours de la fermentation et l'alcool formé; comme un certain nombre de molécules d'acétaldéhyde donnent autre chose que de l'alcool, on doit trouver plus de molécules K de CO<sub>2</sub> que de molécules d'alcool A, et la différence K—A est donnée par:

9. 
$$K - A = a + 2b + 2m + 2s + 3c + h$$

Cette dernière équation est particulièrement difficile à vérifier, car la différence K-A est de l'ordre de 2% de K ou A; pour mesurer cette différence d'une façon utile, il faudrait doser K et A à  $1/10000^{\rm ème}$  près, ce qui présente des difficultés techniques considérables, aussi bien pour le  $CO_2$  (qui est très soluble dans l'eau) que pour l'alcool (qui est souillé d'huiles de fusel).

Il faut remarquer qu'au début de la fermentation, la différence K — A est bien supérieure à 2%. Il est commode d'exprimer K — A en fonction du glycérol g, en partant de l'équation 8.

IO. 
$$K - A = g + b - (a + 3s + 6c)$$

L'ordre de grandeur du phénomène est donné par la valeur de g; or, comme nous le verrons plus loin, au début d'une fermentation, g représente 20 ou 30%, voire plus, si l'on se rapproche du début de la fermentation, des molécules de sucre fermentées; on doit donc mesurer aisément K — A au début de la fermentation. La solubilité du CO<sub>2</sub> introduit une cause d'erreur grave, qui a fait croire à certains expérimentateurs qu'il se formait au début de la fermentation plus d'alcool que de CO<sub>2</sub>. En réalité, il ne peut pas se former une molécule d'alcool sans décarboxylation, et libération de CO<sub>2</sub>; l'inverse n'est pas vrai; il peut apparaître du CO<sub>2</sub>, sans libération d'alcool, par exemple, lorsqu'il se fait de l'acétaldéhyde, de l'acétylméthylcarbinol ou du butylèneglycol.

## 3. Méthodes d'addition ou de soustraction d'acétaldehyde

Les hypothèses faites précédemment ont été démontrées par Peynaud, non seulement par l'analyse d'un grand nombre de fermentations produites en milieu stérile par des levures pures et sélectionnées, mais encore par l'analyse de fermentations en milieux modifiés, et modifiés de deux façons:

- a) par addition progressive d'acétaldéhyde au milieu<sup>6</sup>, on augmente les quantités d'acide acétique, d'acide succinique, de butylèneglycol, qui se forment; on double ces quantités; les 3 corps se comportent de la même façon.
- b) par addition progressive de dimédon au milieu, on diminue la quantité d'acétaldéhyde libre, on "capture" l'acétaldéhyde, et on diminue dans des proportions considérables les trois corps qui en dérivent; on peut réduire l'acide acétique formé au 1/5 de sa valeur dans le témoin.

A côté de ces résultats prévus, des notions nouvelles, les unes attendues, les autres complètement inattendues, ont apparu.

## 4. Rôle du milieu et de la race

Deux notions nouvelles et non surprenantes ont été apportées:

- I. Les proportions d'acides acétique et succinique, et de butylèneglycol, par rapport au glycérol formé, varient beaucoup en fonction du milieu, non seulement du p<sub>H</sub>, mais encore de beaucoup d'autres facteurs (Genevois, Peynaud, Ribereau-Gayon<sup>7</sup>.)
- 2. Dans un même milieu (jus de raisin filtré et stérilisé) les diverses races de levure se comportent très différemment; il est commode de considérer le rapport  $\frac{a}{s}$  de l'acide acétique à l'acide succinique, et le rapport  $\frac{b}{g}$  du butylèneglycol au glycérol. Le rapport  $\frac{a}{s}$  varie de 0.5 à 3, le rapport  $\frac{b}{g}$  de 0.04 à 0.12 (PEYNAUD, RIBEREAU-GAYON<sup>5</sup>).

Ainsi la fermentation alcoolique, qui, d'après des dosages simples d'alcool et de CO<sub>2</sub>, varie très peu en fonction du milieu et de la race de levure, est au contraire une fonction très sensible du milieu et de la race, si l'on considère les produits accessoires issus de la dismutation de l'acétaldéhyde.

Acide acétique, butylèneglycol, acétylméthylcarbinol sont des éléments très importants de l'appréciation des vins, de sorte que nous saisissons comment des levures différentes peuvent donner des vins différents à partir d'un même moût.

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## 5. La réduction de l'acide acétique

Une notion nouvelle et tout à fait inattendue a été apportée par Peynaud¹: l'acide acétique peut à la fois apparaître et disparaître au cours d'une même fermentation; il se forme en quantités relativement grandes au début, puis sa formation s'arrête, et on assiste à la disparition de proportions importantes (parfois les ²/₃) de l'acide acétique formé (Fig. 2). L'acidé acétique est peut-être réduit en alcool, car les acides propionique et butyrique donnent un peu d'alcools propylique et butylique. Corrélativement, il apparaît dans le milieu de l'acide succinique. Tout se passe, au point de vue du bilan, comme si l'on avait la réaction.

3CH<sub>3</sub>CHO + CH<sub>3</sub>CO<sub>2</sub>H + H<sub>2</sub>O = 
$$CO_2H.CH_2CH_2CO_2H + 2CH_3CH_2OH$$

On peut encore supposer la réaction

12. 
$$CH_3CHO + 2CH_3CO_2H = CH_3CH_2OH + CO_2HCH_2CH_2CO_2H$$

Comme 1 molécule d'acide acétique correspond à 2 molécules d'acétaldéhyde, d'après (2), rien n'est changé au bilan 5, quelle que soit l'hypothèse adoptée.

L'équation (II) laisse prévoir que la quantité d'acide acétique qui sera réduite sera toujours inférieure à la quantité d'acide succinique formée. Dans la fermentation d'une molécule de glucose (ISO g) il se forme de 4 à 6 millimolécules d'acide succinique; effectivement, la réduction de l'acide acétique ne dépasse pas 5 millimolécules par litre, et cela lorsque l'on ajoute un excès (I2.4 millimolécules) d'acide acétique au début de la fermentation. Lorsque la levure réduit son propre acide acétique, la quantité réduite ne dépasse pas 3 millimolécules.

Ce phénomène de réduction dépend du milieu; si l'on ajoute un sel de cuivre au milieu, 20 mg par exemple, la réduction de l'acide acétique est empêchée; le cuivre forme des complexes avec la cystéine et la glutathion, et c'est peut-être là le mécanisme de son action. Si l'on ajoute au contraire de la cystéine au milieu, le maximum d'acide acétique et la teneur finale en acide acétique sont nettement plus faibles (Fig. 3).

Ce phénomène dépend de la levure: il est des levures réductrices, qui font disparaître les  $^2/_3$  de l'acide acétique qu'elles produisent; on observe en fin de fermentation un  $p_H$  bas, de l'ordre de 9. Il est au contraire des levures sans action sur l'acide acétique qu'elles forment; ces levures donnent au milieu où elles fermentent, un  $p_H$  relativement

TABLEAU PRODUITS FORMÉS AU COURS D'UNE

| Temps                 | Sucres                          | Alcool                       | Glycérol                  |                                | Cati                           | ons                             |                          |
|-----------------------|---------------------------------|------------------------------|---------------------------|--------------------------------|--------------------------------|---------------------------------|--------------------------|
| Jours                 | g                               | degrés                       | Mill.                     | Acid.                          | Alcal.                         | NH <sub>3</sub>                 | Somme                    |
| 0<br>I<br>2<br>3<br>8 | 166<br>134<br>86<br>56.5<br>6.5 | 2°.2<br>5°.0<br>6°.8<br>9°.9 | 0<br>53<br>58<br>67<br>82 | 95<br>104<br>107<br>107<br>112 | 61<br>61.7<br>62<br>62<br>62.4 | 1.9<br>0.4<br>0.4<br>0.3<br>0.3 | 158<br>166<br>169<br>169 |
| Bilan                 |                                 |                              |                           | + 17                           | + 1.4                          | 1.6                             | + 16.8                   |

Cation et anions sont exprimés en milliéquivalents par litre. Aux cations: Acid. signifie Acidité de titration (à p<sub>H</sub> 7.5). Alcal. signifie Alcalinité des cendres. élevé de 12 à 16. Il est des levures ayant une action modérée sur l'acide acétique; on observe alors des p<sub>H</sub> de 10 à 11. Des études ultérieures préciseront les substances qui sont à l'origine de ces différences.

## II. ÉTUDE EXPÉRIMENTALE DES PRODUITS FORMÉS AU COURS DE LA FERMENTATION

## 1. Le départ de la fermentation

Le Tableau I, emprunté à E. Peynaud (1947) montre l'évolution d'une fermentation d'un moût du cépage de raison rouge petit Verdot, à 25°. Le moût a été stérilisé au moment de la récolte, conservé en bouteilles, ensemencé ensuite au laboratoire d'une levure pure particulièrement réductrice. On a supposé que le bitartrate ne précipitait pas, et le bilan a été calculé en rajoutant le bitartrate précipité à celui subsistant dans le milieu. Les équilibres de précipitation de bitartrate sont en effet fort longs à atteindre. Des échantillons de chaque stade de fermentation ayant été prélevés et conservés, un tableau a pu être dressé en tenant compte du bitartrate précipité, l'équilibre de solubilité ayant été réalisé (Peynaud).

La fermentation est partie rapidement, de sorte que, au bout de 24 heures, 32 g de glucose et de lévulose avaient déjà fermenté, ce sucre représente 178 millimolécules; il a engendré 53 millimolécules de glycérol; 30% du sucre ont donc suivi la voie de la fermentation glycéropyruvique. Si l'on pouvait suivre le sort des 20 premières millimolécules de sucre fermentées, il est probable que la fermentation glycéropyruvique prédominerait.

En même temps que le glycérol, on voit apparaître 2 millimolécules d'acide lactique, ce qui montre qu'au départ de la fermentation alcoolique, 1% au moins du sucre emprunte la voie de la fermentation lactique. Cette proportion s'élèvera sensiblement à la fin: les 50 derniers grammes — 280 millimolécules — engendreront 5 millimolécules d'acide lactique, ce qui représente 1.8% du sucre consommé. Si l'acide lactique était dû à des bactéries, on n'observerait pas ce phénomène: les bactéries lactiques sont toujours inhibées par l'alcool, en sorte que l'on verrait se former plus d'acide lactique au début qu'à la fin de la fermentation.

Outre l'acide lactique, il apparaît au début de la fermentation de l'acide acétique, 5.5 milliéquivalents, de l'acide succinique, 2.9 milliéquivalents, de l'acide citrique

FERMENTATION ALCOOLIQUE

|       |        |       | Anie  | ons     |       |         |        |
|-------|--------|-------|-------|---------|-------|---------|--------|
| Tart. | Mal.   | Citr. | Acét. | Succin. | Lact. | Phosph. | Somme  |
| 101   | 46.5   | 3.5   | 1.0   | О       |       | 1.5     | 153    |
| 101   | 45     | 4.0   | 6.5   | 2.9     | 2.0   | 1.5     | 163    |
| 101   | 45     | 4.2   | 5.5   | 7.1     | 3.2   | 1.5     | 167    |
| 101   | 43.5   | 4.5   | 5.0   | 8.7     | 4.0   | 1.5     | 167    |
| 101   | 41.4   | 4.7   | 3.8   | 11.2    | 9.0   | 1.0     | 172    |
|       | — 5. г | + 1.2 | + 2.8 | + 11.2  | +9    |         | + 19.1 |
|       |        | '     |       |         |       | 1       |        |

o.5 milliéquivalents. Si l'on suit ces acides au cours de la fermentation, on observe l'augmentation progressive des acides succinique et citrique, mais par contre on voit disparaître progressivement l'acide acétique, sur 6.5 milliéquivalents au bout de 24 heures de fermentation, 2.7 disparaissent, et il ne reste finalement que 3.8 milliéquivalents.

En fin de fermentation, il est apparu 11.2 milliéquivalents d'acide succinique, 9 d'acide lactique, 2.8 d'acide acétique, 1.2 d'acide citrique. Considérer l'acidité formée au cours de la fermentation comme due au seul acide succinique est donc ignorer la complexité du phénomène.

## 2. L'acide lactique

Peynaud² a dosé l'acide lactique formé au cours de la fermentation d'un moût de raisin à 156 g de sucre au litre, de  $p_H$  3.26, par 15 levures pures, retirées presque toutes de vins de la Gironde; il a trouvé de 5 à 7 milliéquivalents d'acide lactique formé, ce qui, ramené à 180 g de sucre, représente de 6.0 à 8.5 milliéquivalents.

Il a fait fermenter un moût de raisin à 180 g de sucre du litre, qu'il a ajusté à des  $p_H$  allant de 2.7 à 7.0; une levure de *Fronsac* a donné des quantités d'acide lactique allant de 5.4 à 6.7 milliéquivalents, sans relation avec le  $p_H$ ; une levure de *Saint-Emilion* a donné de 5.1 à 6.3 milliéquivalents, également sans relation avec le  $p_H$ . L'acide lactique est donc un produit très constant de la fermentation par les levures, qui ne varie pratiquement pas entre de larges limites de  $p_H$ .

## 3. Formation et disparition de l'acide acétique

La formation d'acide acétique varie énormément:

- 1. Avec la race de levure.
- 2. Avec les conditions de milieu.

Avec la plupart des levures, l'acide acétique formé passe par un maximum, parfois tout au début de la fermentation, le plus souvent lorsque la moitié ou les deux tiers du sucre ont fermenté. Ce maximum est compris entre 2 et 9 milliéquivalents par litre;

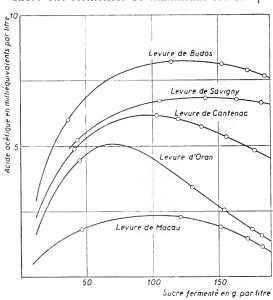


Fig. 1

l'acide acétique en fin de fermentation est compris entre 1 et 8 milliéquivalents, tout cela pour des fermentations suivies dans des jus de raisin filtrés et stériles (Peynaud<sup>1, 2</sup>) (Fig. 1).

Dans les conditions de la vinification normale, la proportion d'acide acétique formée est beaucoup plus élevée, les vins contiennent normalement de 10 à 20 milliéquivalents d'acide acétique que l'on peut attribuer à la fermentation alcoolique, indépendamment de la piqure acétique, ou des traces d'acide acétiques formées dans la fermentation malolactique (Genevois, Peynaud, Ribereau-Gayon<sup>8</sup>).

La formation d'acide acétique en fonction du p<sub>H</sub> présente toujours un

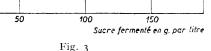
minimum, minimum dont la valeur absolute est très variable selon la race, de 2 à ro milliéquivalents, et qui se produit pour des p<sub>H</sub> allant de 3.5 à 5 (Peynaud<sup>2</sup>) (Fig. 2).

En milieu neutre (p<sub>H</sub> 7), la production d'acide acétique va de 15 à 25 milliéquivalents par litre, selon la race et augmente rapidement avec le p<sub>H</sub>. On tend § 15 vers la "fermentation alcaline" de C. Neuberg. Cu empêche la réduction, la § cystéine favorise la réduction de l'acide acétique (Fig. 3).

## 4. Formation d'acide citrique au cours de la fermentation

La levure forme, en anaérobiose, une petite quantité d'un acide ayant tous les caractères analytiques de l'acide citrique. Cet acide a été recherché dans la fermentation d'un moût de raison à 166 g de sucres, contenant déjà 4 milliéquivalents d'acide citrique au litre. 7 levures différentes ont donné des quantités d'acide citrique supplémentaires allant de I à 2 milliéquivalents par litre. On peut se demander si cet acide citrique ne provient pas

Acide acétique en milliéquivalents par litre o 20 mg de Cui Témoin



0.5 g de Cysteine

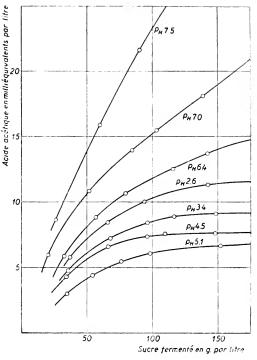


Fig. 2

de l'acide malique présent normalement dans le moût. La levure détruit en effet de 10 à 20% de l'acide malique présent, en passant par le stade d'acide oxalacétique; or l'acide oxalacétique réagit biochimiquement avec l'acide pyruvique pour donner de l'acide citrique. Mais l'expérience montre que les quantités d'acide citrique formées sont indépendantes des quantités d'acide malique présentes, ou transformées.

Si l'on fait fermenter 5 fois un même milieu, auquel on ajoute après chaque fermentation du sucre après élimination de l'alcool, on observe la formation de quantités régulièrement croissantes d'acide citrique, de 1.5 milliéquivalents à chaque opération (Peynaud<sup>2, 10</sup>).

# III. INFLUENCE DES ADDITIONS OU SOUSTRACTIONS D'ACÉTALDEHYDE SUR LA FERMENTATION ALCOOLIQUE

## 1. Addition d'acétaldehyde

Il est impossible d'ajouter brutalement de l'acétaldéhyde au milieu de fermentation, car l'éthanal est toxique pour la levure à des doses supérieures à M/100. Il faut ajouter l'éthanal lentement, à raison de 0.2 g par jour et par litre, par exemple, pendant 10 jours; la fermentation est simplement un peu ralentie. La levure arrive ainsi à transformer en 10 jours 2 g d'acétaldéhyde, pour 190 g de sucre fermenté, ce qui représente 5% du nombre des molécules de sucre fermentées. Cela suffit pour modifier profondément les quantités de produits secondaires de la fermentation (Genevois, Peynaud, Ribéreau-Gayon?).

L'expérience a été répétée avec deux levures: une levure de vin rouge typique, levure de Pomerol, une levure industrielle de boulangerie.

Les résultats sont portés sur le Tableau II, dans les deux cas, les trois produits secondaires de la fermentation, acides acétique et succinique, et butylèneglycol, augmentent massivement.

TABLEAU II

ADDITION D'ACÉTALDÉHYDE À DEUX FERMENTATIONS (JUS DE RAISON À 190 g DE SUCRE)

MILLIMOLÉCULES POUR 10 LITRES DE MILIEU FERMENTÉ (SAUF POUR L'ALCOOL)

|             |                     |              |            | Acides    |           |                              |                    |                     |          |                             |
|-------------|---------------------|--------------|------------|-----------|-----------|------------------------------|--------------------|---------------------|----------|-----------------------------|
| Levure      | Aldehyde<br>ajoutée | Alcool       | Glycérol   | Acétique  | Succin.   | Acétyl<br>méthyl<br>carbinol | Butylène<br>glycol | Aldéhyde<br>restant | $\Sigma$ | $\frac{\Sigma}{\mathrm{g}}$ |
|             | Н                   |              | g          | a         | s         | m                            | ь                  | h                   |          |                             |
| Pomerol     | 0<br>502            | 11°0<br>11°2 | 57º<br>500 | 92<br>144 | 33<br>73  | 0.2                          | 34<br>93           | 8<br>69             |          |                             |
|             |                     | + 0.2        | - 70       | + 52      | +40       | 1.0+                         | + 59               | + 61                | 363      | 83                          |
|             |                     |              |            |           | 51        |                              |                    |                     |          |                             |
| Boulangerie | 0<br>502            | 10°S         | 580<br>510 | 37<br>109 | 0.6<br>84 | 0.6                          | 42<br>119          | 8 28                |          |                             |
|             |                     | + 0.2        | — 70       | + 72      | +33       | + 9.7                        | 77                 | + 20                | 406      | 85                          |

L'expérience consiste à comparer une fermentation de jus de raison (190 g de sucre réducteur par litre,  $p_H=3.17$ ) recevant 0.2 g d'acétaldéhyde par jour, à une fermentation témoin, marchant parallèlement, avec la même levure.

La levure de Pomerol fait passer l'acide succinique de 33 à 73 millimolécules pour 10 litres, l'acide acétique de 92 à 144, le butylèneglycol de 34 à 93, l'acide succinique augmente de 120%, le butylèneglycol de 160%, 45% de l'acétaldéhyde ajoutée sont passés à former de l'acide succinique et de l'alcool selon l'équation 4. La levure de boulangerie "travaille" moins l'acétaldéhyde à l'état d'acide succinique, néanmoins 34% de l'aldéhyde se retrouve encore dans l'acide succinique et l'alcool correspondant. Chaque levure possède sa façon personelle de distribuer l'acétaldéhyde entre les acides acétique et succinique, le butylèneglycol et l'acétylméthylcarbinol.

### 2. Addition de dimédon

Le moyen le moins brutal de soustraire de l'acétaldéhyde au milieu de fermentation, consiste à ajouter du dimédon (Peynaud²). Le dimédon étant très peu soluble dans l'eau, il faut l'ajouter en solution alcoolique; pour qu'il reste convenablement en solution, il faut que la teneur initiale du milieu en alcool soit de  $4^{\circ}$  environ; pour qu'il réagisse assez vite avec l'aldéhyde, il faut que le  $p_H$  du milieu soit d'au moins 4 et de préférence 6. Le Tableau III porte deux fermentations, réalisées dans du jus de raisin à  $4^{\circ}$  d'alcool, à  $p_H$  4.0 et 6.5, chaque fois avec et sans dimédon. Sans dimédon, l'acide acétique apparaît dès les premiers jours de fermentation, puis n'augmente plus que lentement; avec dimédon, la formation d'acide acétique est très faible au début, et se poursuit lentement, au cours de toute la fermentation.

TABLEAU III action du dimédon sur la formation d'acide acétique jus de raisin à 4° d'alcool et à 1% de dimédon

|              |                | Témoin                 |                                |                | + 1% dimédo            | n                              |
|--------------|----------------|------------------------|--------------------------------|----------------|------------------------|--------------------------------|
| PH du milieu | Temps<br>Jours | Sucre<br>fermenté<br>g | Acide<br>Acétique<br>Milliéqu. | Temps<br>Jours | Sucre<br>fermenté<br>g | Acide<br>Acétique<br>Milliéqu. |
| 4.0          | 3              | 56                     | 3.6                            | 5              | 36                     | 0.9                            |
|              | 4              | 96                     | 4.1                            | 7              | 68                     | 1.2                            |
| ŀ            | 5              | 125                    | 4.3                            | 12             | 133                    | 2.2                            |
| 6.5          | 3              | 48                     | 9.3                            | 3              | 26                     | 1.0                            |
|              | 5              | 105                    | 17.3                           | 5              | 89                     | 2.9                            |
|              | 6              | 125                    | 20.3                           | 6              | 105                    | 4.3                            |

A  $p_H$  4, le dimédon réagissant lentement, la formation d'acide acétique est réduite à la moitié de sa valeur normale. A  $p_H$  6.5, le dimédon réagissant mieux, l'acide acétique est réduit à 21% de la valeur du témoin.

## IV. BILAN DES PRODUITS SECONDAIRES DE LA FERMENTATION

## 1. Milieu constitué par du jus de raison stérilisé

Sur un même jus de raisin à 190 g de sucre au litre, de p<sub>H</sub> = 3.17, ont fermenté 29 levures différentes de provenances très variées: 16 levures de vins rouges de la Gironde, 2 levures ayant poussé spontanément sur des jus de raisins concentrés, 1 levure de vin blanc, 1 levure de boulangerie, et 9 levures de vins suisses (Peynaud<sup>12</sup>). Les dosages ont porté sur les substances figurant dans les bilans (5) et (6), savoir: glycérol (g), acides acétique (a) et succinique (s), acétylméthylcarbinol (m), 2–3 butylèneglycol (b), acétaldéhyde (h). La production de glycérol a relativement peu varié d'une levure à l'autre: 52 à 75 millimolécules par litre; l'acide acétique a varié de 3 à 12, l'acide succinique de 4.8 à 9, l'acétylméthylcarbinol de 0.02 à 0.11, le butylèneglycol de 3 à 6, l'acétaldéhyde de 1,5 à 4, le tout en millimolécules. Malgré ces grandes variations, dues à la diversité des races physiologiques de levure, le bilan 6 se vérifie aux erreurs d'expériences près (Tableau IV) (Genevois, Peynaud, Ribéreau-Gayon<sup>2</sup>). Si l'on appelle

 $\sum la somme: \qquad \sum = 5s + 2a + 2m + b + h$ 

on observe que le rapport  $\frac{\sum}{g}$  est compris entre 0.82 et 0.96, les chiffres les plus fréquents étant voisins de 0.90.

TABLEAU IV

BILAN DES PRODUITS SECONDAIRES DE LA FERMENTATION
FERMENTATIONS SUR JUS DE RAISIN STÉRILE (J) ET SUR SOLUTION DE SACCHAROSE (S)

MILLIMOLÉCULES POUR 10 LITRES

| Levure    | Type        | Milieu | g          | a         | s                | m   | b        | h        | Σ                                    | $100\frac{\Sigma}{g}$ | $\frac{a}{s}$ | 1000-b    |
|-----------|-------------|--------|------------|-----------|------------------|-----|----------|----------|--------------------------------------|-----------------------|---------------|-----------|
| Margaux   | Succinogène | J<br>S | 650<br>840 | 43<br>153 | 77<br>68         | 0.2 | 36<br>85 | 37<br>10 | 544<br>741                           | 84<br>88              | 0.56          | 55<br>101 |
| Boulanger | Id. glycol. | J<br>S | 640<br>820 | 37<br>153 | 80<br>65         | 0.7 | 55<br>83 | 26<br>10 | 555<br>7 <sup>8</sup> 4              | 86<br>89              | 0.7<br>2.4    | 84        |
| La Tresne | Equilibré   | J<br>S | 610<br>780 | 78<br>220 | 70<br>40         | 0.3 | 33<br>84 | 33<br>7  | 57 <sup>2</sup><br>73 <sup>1</sup>   | 93<br>92              | 1.1<br>5·5    | 55<br>106 |
| Malvoisie | Id. glycol. | J<br>S | 600<br>750 | 59<br>160 | 60<br><b>5</b> 9 | 0.9 | 57<br>71 | 27<br>4  | 502<br>640                           | 83<br>85              | 1.0<br>2.7    | 95<br>94  |
| Pauillac  | Acétogène   | J<br>S | 630<br>750 | 84<br>218 | 58<br>38         | 0.4 | 34<br>77 | 3 I<br>5 | 523<br>708                           | 83<br>94              | 1.4<br>5·7    | 54<br>102 |
| Bonarda   | Id. glycol. | J<br>S | 610<br>790 | 100       | 48<br>36         | 0.7 | 56<br>59 | 31<br>8  | 5 <sup>2</sup> 7<br>7 <sup>1</sup> 3 | 86<br>90              | 2.0<br>6.5    | 92<br>75  |

Glycol. = abréviation pour "butylèneglycologène"

Il reste donc un ou plusieurs constituants, dérivant de l'acétaldéhyde, qui restent à déterminer, mais ils ne représentent pas plus de 10 à 15% de l'acétaldéhyde dérivant de la fermentation glycéropyruvique.

Il est commode de considérer le rapport de l'acide acétique à l'acide succinique  $\frac{a}{e}$  et le rapport du butylèneglycol au glycérol,  $\frac{b}{g}$ ; le rapport  $\frac{a}{s}$  varie de 0.4 à 2.1, selon les levures, le rapport  $\frac{b}{g}$  varie de 0.048 à 0.095, ces rapports permettent un classement physiologique des levures, en levures succinogènes  $\left(\frac{a}{s} < 0.75\right)$  acétogènes  $\left(\frac{a}{s} > 1.25\right)$  et équilibrées (0.75  $<\frac{a}{s} < 1.25$ ); les levures où  $\frac{b}{g} > 0.070$  pourront être considérées comme glycologènes (Peynaud et Ribéreau-Gayon<sup>5</sup>).

On peut répartir les levures dans six catégories physiologiques différentes (Tabl. IV). Ainsi ce travail analytique considérable aboutit à deux résultats: vérifier les hypothèses faites sur l'origine des acides acétique et succinique, et du butylèneglycol, donner une description logique des diverses races de levures.

## 2. Milieu constitué par une solution de saccharose et d'eau de levure

L'expérience précédente, portant sur 29 levures différentes, a été répétée sur une solution de saccharose à 180 g au litre, contenant 10% d'extrait de levure. Le  $p_H$  de ce milieu se stabilisait aux environs de 5. Les mêmes produits que précédemment ont été Bibliographie p. 191/192.

dosés; ils se sont trouvés systématiquement différents (Gevenois, Peynaud, Ribéreau-Gayon<sup>7</sup>).

- 1. le glycérol g augmente de 10 à 40%
- 2. l'acide acétique a est en moyenne 3 fois plus élevé
- 3. l'acide succinique s diminue de 10 à 20%
- 4. le butylèneglycol b double généralement
- 5. l'acétylméthylcarbinol m est en quantités du même ordre
- 6. l'acétaldéhyde h tombe au quart de sa concentration.

Par contre, l'équation (6) se vérifie comme précédemment; le mécanisme de la fermentation est le même, mais la distribution de l'acétaldéhyde entre les divers produits de fermentation est différente. Le rapport  $\frac{a}{s}$ , au lieu de varier de 0.4 à 2.1 varie de 2.0 à 6.5; le rapport  $\frac{b}{g}$  varie de 0.07 à 0.11 (Tableau IV).

Les différentes catégories de levures, caractérisées par leur fermentation sur jus de raisin filtré et stérile, présentent sur milieu au saccharose d'autres constantes, comme il est normal; chez toutes les levures, le rapport  $\frac{a}{s}$  augmente considérablement, les levures succinogènes présentent des rapports  $\frac{a}{s}$  de 2 à 3, au lieu de 0.4 à 0.75; les levures acétogènes présentent des rapports  $\frac{a}{s}$  de 5 à 7 au lieu de 1.25 à 2. Les levures dites "équilibrées", au lieu d'un  $\frac{a}{s}$  voisin de 1, donnent pour  $\frac{a}{s}$  des valeurs échelonnées de 3 à 5.5. Ces trois catégories de levures se retrouvent donc sans difficulté.

Les levures qui présentaient des rapports  $\frac{b}{g}$  faibles, de 0.04 à 0.07, présentent des rapports  $\frac{b}{g}$  voisin de 0.10. Les levures caractérisées comme glycologènes précédemment, avec un  $\frac{b}{g}$  déjà voisin de 0.10, gardent sensiblement la même valeur pour le rapport  $\frac{b}{g}$ , comme s'il y avait un "plafond" pour la formation de butylèneglycol. Le rapport  $\frac{b}{g}$  oscille, comme précédemment, entre 0.82 et 0.95. Il a donc là une

Le rapport  $\frac{\sum}{g}$  oscille, comme précédemment, entre 0.82 et 0.95. Il a donc là une véritable "constante" biologique, indépendante dans une large mesure de la race de la levure et de la nature du milieu fermenté.

## 3. Fermentation dans les vins

La fermentation dans les vins est rarement une fermentation alcoolique pure; dans à peu près tous les vins non sulfités ni additionnés d'alcool, l'acide malique est transformé en acide lactique, par fermentation malolactique due à des bactéries spéciales; d'après Peynaud, qui a soigneusement étudié ce type de fermentation à Bordeaux, il apparaît, non seulement de l'acide lactique, à raison d'une molécule par molécule d'acide malique détruit, mais encore un peu d'acide acétique, de 1 à 7 milliéquivalents par litre, qui semble provenir d'une autre source. Les chiffres les plus fréquents pour l'acide acétique ainsi formé vont de 2 à 4 milliéquivalents. Il semblerait donc que le bilan indiqué par l'équation (6) ne doive plus se vérifier. Cependant, si l'on considère Bibliographie p. 191/192.

TABLEAU V
BILAN DES PRODUITS SECONDAIRES DE LA FERMENTATION DANS LES VINS ROUGES ET BLANCS

| Année                                                                | Туре  | Vin                                                                                | g                                                                  | a                                                              | s                                                  | m                                      | b                                                        | h                    | Σ                                                                  | $\left  \frac{\Sigma}{g} \right $                  | <u>a</u><br>s                                                 | 1000 b                                                       |
|----------------------------------------------------------------------|-------|------------------------------------------------------------------------------------|--------------------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------|----------------------------------------|----------------------------------------------------------|----------------------|--------------------------------------------------------------------|----------------------------------------------------|---------------------------------------------------------------|--------------------------------------------------------------|
| 1945<br>1945<br>1944<br>1944<br>1943<br>1943<br>1946<br>1946<br>1946 | Rouge | Pomerol Blaye Moulis St. Emilion Listrac Bourg Tuchan Tautavel Tautavel Frontignan | 850<br>830<br>804<br>654<br>862<br>890<br>590<br>730<br>770<br>250 | 112<br>163<br>122<br>122<br>174<br>200<br>66<br>90<br>99<br>53 | 90<br>71<br>85<br>51<br>84<br>71<br>65<br>74<br>71 | 0.9<br>0.5<br>1.1<br>2.2<br>1.1<br>0.5 | 81<br>79<br>65<br>48<br>70<br>71<br>64<br>92<br>94<br>32 | 16<br>36<br>35<br>17 | 757<br>761<br>736<br>551<br>840<br>827<br>537<br>678<br>682<br>235 | 89<br>90<br>91<br>85<br>98<br>93<br>91<br>93<br>88 | 1.24<br>2.16<br>1.4<br>2.4<br>2.1<br>2.8<br>1.0<br>1.2<br>1.4 | 95<br>93<br>81<br>73<br>81<br>80<br>108<br>126<br>122<br>128 |

des vins jeunes, de un à deux ans d'âge, des vins pasteurisés, des vins "vinés" c'est-à-dire additionnés d'alcool au cours de la fermentation, pour garder du sucre, le bilan (6) se vérifie presque toujours.

Par exemple, sur 20 échantillons de vins rouges de la Gironde analysés par Peynaud<sup>8</sup> au printemps 1946, 18 présentent des rapports  $\frac{\sum}{g}$  normaux, allant de 0.82 à 0.98 et 2 seulement des rapports supérieurs à 1, par suite d'un excès d'acide acétique.

L'expérience montre que le rapport  $\frac{a}{s}$  est dans un vin rouge issu de la fermentation de la macération de la totalité de la baie de raison fraîche, très différent de ce qu'il est dans la fermentation d'un jus de raison filtre et stérilisé.

Dans les vins rouges, le rapport  $\frac{a}{s}$  s'est toujours trouvé compris entre 1 et 3, en éliminant les échantillons contenant visiblement de l'acide acétique dû à une fermentation acétique. La valeur absolue de a va de 10 à 20 milliéquivalents, alors que sur jus de raison stérile il va de 3 à 12; les levures françaises donnent même pour a des valeurs comprises entre 3 et 8. Même en tenant compte de l'acide acétique de la fermentation malolactique, 2 à 4, il est clair que la fermentation due aux levures se fait dans le moût naturel autrement que dans nos flacons, et que le rendement en acide acétique est au moins doublé.

Dans le cas des vins blancs du midi³, très riches en sucre, mutés à l'alcool en cours de fermentation, ce qui empêche l'action des bactéries malolactiques, le rapport  $\frac{\sum}{g}$  s'est trouvé compris entre 0.88 et 0.94. Le cas du muscat de Frontignan, muté après fermentation du quart à peine de son sucre, est très intéressant; le rapport  $\frac{a}{s}$  est élevé, 3.3, comme il est normal au départ de la fermentation; le rapport  $\frac{b}{g}$  est aussi remarquablement élevé, 0.13; le rapport  $\frac{\sum}{g}$  est normal, 0.94.

#### CONCLUSIONS

La détermination des produits secondaires de la fermentation, glycérol, acides acétique et succinique, acétylméthylcarbinol, butylèneglycol et acétaldéhyde présente donc un grand intérêt:

1. Toutes ces substances sont des produits normaux de la fermentation alcoolique.

2. Elles proviennent d'une fermentation glycéropyruvique, qui prédomine au départ de la fermentation, mais se poursuit durant toute la destruction du sucre.

3. Elles obéissent à l'équation (6).

4. Les rapports de l'acide acétique à l'acide succinique, du butylèneglycol au glycérol, varient en fonction du moment de la fermentation, de la race de levure, enfin de la nature du milieu fermenté (p<sub>H</sub>, etc. . .).

5. Ces rapports peuvent, dans un milieu donné, servir à caractériser des races de levures.

- 6. L'acide acétique suit au cours de la fermentation une évolution compliquée, qui le fait apparaître, puis disparaître.
- 7. Dans les fermentations naturelles (vin), la considération de l'équation (6) permet de caractériser certaines altérations bactériennes graves.

#### CONCLUSIONS

Determination of the secondary products of alcoholic fermentation: glycerol, acetic acid, succinic acid, acetyl methyl carbinol, butyleneglycol and acetaldehyde, is of great interest, for:

1. All these compounds are normal products of alcoholic fermentation.

2. They arise from a glycero-pyruvic fermentation, which dominates in the beginning of the fermentation, but perseveres during the destruction of all the sugar.

3. They agree with equation (6).

4. The relation between acetic acid and succinic acid, as between butyleneglycol and glycerol, depends upon the phase of the fermentation, the strain of yeast, and finally also upon the nature of the medium in which fermentation takes place (p<sub>H</sub>, etc.).

5. In a given medium these relations can serve to characterize the strains of yeasts.

- 6. During the fermentation acetic acid is subject to a complicated evolution, which causes it to appear and then to disappear again.
- 7. In natural fermentations (wine) a consideration of equation (6) enables the characterization of certain serious bacterial changes.

## SCHLUSSFOLGERUNGEN

Die Bestimmung der Nebenprodukte der alkoholischen Gärung: Glycerin, Essigsäure, Bernsteinsäure, Acetylmethylcarbinol, Butylenglykol und Acetaldehyd ist aus folgenden Gründen wichtig:

1. Alle diese Verbindungen sind normale Produkte der alkoholischen Gärung.

2. Sie stammen aus einer Glycero-Brenztraubensäure-Gärung, die zu Beginn der Gärung vorherrscht, aber während der ganzen Zersetzung des Zuckers fortdauert.

3. Sie sind im Einklang mit Gleichung (6).

- 4. Das Verhältnis Essigsäure/Bernsteinsäure und Butylenglykol/Glycerin hängt von der Phase der Gärung, von dem benutzten Hefestamm und endlich von der Natur des Milieus ab, in dem die Gärung stattfindet (p<sub>H</sub>, usw.).
- 5. In einem bestimmten Milieu können diese Verhältnisse zur Charakterisierung der Heferasse dienen.
- 6. Die Essigsäure ist während der Gärung einem komplizierten Prozess unterworfen, durch den sie zuerst auftritt und dann wieder verschwindet.
- 7. Bei natürlichen Gärungen (Wein) kann man durch Betrachtung der Gleichung (6) gewisse ernste bakterielle Veränderungen charakterisieren.

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<sup>5</sup> PEYNAUD ET RIBÉREAU-GAYON, Ibidem, 224 (1947) 1388.

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## TRIOSEPHOSPHORSÄURE ALS INTERMEDIÄRPRODUKT BEI DER ZUCKERGÄRUNG MIT INTAKTER HEFE

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Der heute allgemein anerkannte Abbauweg bei der Vergärung von Zucker zu Alkohol über phosphorylierte Intermediärprodukte wurde für den zellfreien Fermentextrakt (Macerationssaft) als Schema im Jahre 1933 von O. Meyerhof¹ im Princip das erstemal aufgestellt und im Jahre 19352 und 19373 ergänzend erweitert. Bei diesem Schema wird zwischen Initialphase und stationärer Phase unterschieden. In der Ergänzung von 1937 findet sich auch eine Erklärung für die Entstehung der Harden-Young'schen Gärungsphase im zellfreien Macerationssaft; aber gerade die Bildung von Hexoseestern ist für die Gegner dieses Schemas immer ein Punkt der Kritik, nach der der Abbau des Zuckers in der lebenden Zelle anders verlaufen sollte, weil bei ihr diese Ester als Gärungszwischenprodukte nicht nennenswert sich anhäufen und nachzuweisen sind. In einem wirklichen stationären Zustand ist nun die Anreicherung eines Intermediärproduktes nicht zu erwarten, da jeder Fall dieser Art eine Zustandsänderung voraussetzt, sei es Alterung, Hemmung durch Gifte oder Erschöpfung von Nährstoffen u.s.w. Auch die Initialphase der Gärung ist eine solche Zustandsänderung. Im folgenden soll die vorübergehende Anreicherung einer Triosephosphorsäure als Intermediärprodukt bei der Angärung von intakter Hefe beschrieben werden.

## METHODIK

Triosephosphorsäure wurde nachgewiesen als Milchsäure, welche durch alkalische Verseifung bei Zimmertemperatur nach der von O. Meyerhof und K. Lohmann<sup>3a</sup> beschriebenen Reaktion entsteht: Triosephosphat → Milchsäure + Phosphat. Die so gebildete Milchsäure in den Gäransätzen wurde folgendermassen bestimmt: 20 ml Gärlösung wurden filtriert, schwach Phenolphtalein-alkalisch zur Trockne verdampft, 2 mal mit Wasser aufgenommen und nochmals verdampft, im Schwefelsäureexsiccator über Nacht getrocknet; dann in 20 ml Wasser gelöst, und zur Verseifung der Triosephosphorsäure mit I ml 25% Natronlauge versetzt. Nach 10 Minuten Stehen bei Zimmertemperatur wurde mit I ml 25% Salzsäure neutralisiert, mit CuSO<sub>4</sub>-Ca(OH)<sub>2</sub> gefällt, ein aliquoter Teil abgenommen und in der üblichen Weise nach Friedemann, Contonio und Shaffer4 die Milchsäure bestimmt. Als Hefe wurde Weinhefe Steinberg aus Geisenheim am Rhein oder Weinhefe Oppenheimer Kreuz aus Oppenheim am Rhein verwandt.

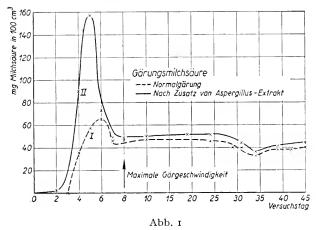
Natürliche Nährsubstrate waren Traubenmoste oder Moste aus anderen Früchten. Als synthetische Nährlösung diente ein modifizierter Gäransatz nach Henneberg<sup>5</sup>, bestehend aus 15% Rohr-

zucker, 0.2% Pepton, 0.5% KH<sub>2</sub>PO<sub>4</sub>, 0.2% MgSO<sub>4</sub>. p<sub>H</sub> 4.8. Um die Triosephosphorsäure zu isolieren und fernerhin die bereits vorgebildete Milchsäure von derjenigen zu unterscheiden, die erst durch alkalische Verseifung entsteht, wurden die Gäransätze durch Fällung mit Bariumacetat und Alkohol fraktioniert. Beim Fraktionieren wurden 20-50 ml der mit Trichloressigsäure enteiweissten Gäransätze mit Bariumacetat versetzt und bei schwach lackmussaurer Reaktion mit 3 Teilen Alkohol gefällt. Der Niederschlag wurde mit Alkohol-Aether ge-

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waschen und im Schwefelsäureexsiccator über Nacht getrocknet. Darauf wurde im ursprünglichen Volumen Wasser mit einigen Tropfen 2 n Salzsäure gelöst, Ba mit Na $_2$ SO $_4$ ausgefällt, mit 1 ml $_25\%$ iger Natronlauge bei Zimmertemperatur verseift; dann mit 1 ml $_25\%$ iger Salzsäure neutralisiert und nach CuSO $_4$ –Ca(OH) $_2$ -Behandlung in einem aliquoten Teil die Milchsäure bestimmt. Ein entsprechender Anteil wurde vor der CuSO $_4$ –Ca(OH) $_2$ -Behandlung zur  $P_2O_5$ -Bestimmung der Triosephosphorsäure abgenommen und als anorganisches Phosphat nach der Verseifung mit normaler Natronlauge bestimmt. Die kolorimetrische Phosphorsäurebestimmung nach Lohmann und Jendrassik $^6$  wurde in einem lichtelektrischen Kolorimeter nach Havemann mittels einer Eichkurve vorgenommen. Zunahme des anorganischen Phosphats nach alkalischer Verseifung entspricht der Triosephosphorsäure.

In der Mutterlauge der Bariumfällung konnte die wirkliche Milchsäure, d.h. diejenige, die nicht erst durch alkalische Verseifung entstanden ist, nach Ba-Fällung mit Natriumsulfat in der oben angegebenen Weise ebenfalls bestimmt werden.



#### VERSUCHE

Verfolgt man die Milchsäurebildung eines gärenden Traubensaftes während des ganzen Gärverlaufes, so ist zunächst in der Angärungsphase ein Ansteigen der Milchsäure festzustellen, die bei Beginn der stationären Phase auf einen gleichbleibenden Gehalt bis zum Ende der Gärung abfällt. In der Abb. 1 ist eine derartige Milchsäurebildung bei der normalen Vergärung von 1200 l Traubenmost aus Sylvanertrauben in der Versuchs-

und Forschungsanstalt für Wein- und Obstbau zu Geisenheim aus dem Jahre 1944 aufgezeichnet. Das  $p_H$  dieses Mostes betrug 3.3 und die Kellertemperatur Ende Oktober

9-11°. Kurve I (Abb. 1) zeigt in der Angärungsphase zwischen dem 2. und 8. Tag einen Anstieg der Milchsäure bis zu 65 mg/%, der dann bei einsetzender maximaler Gärgeschwindigkeit auf 46 mg/% absinkt und während und nach beendeter Gärung bei ungefähr 40 mg bleibt bis zum Einsetzen der bakteriellen Säuregärung. Es wurde nun weiter gefunden, dass sich dieser Milchsäureanstieg durch Zusatz eines wässrigen Extraktes aus getrockneten Schimmelpilzen (Aspergillus niger) bedeutend steigern lässt. Nach v. Euler und NIELSEN<sup>7</sup> enthält Aspergillus niger einen wasserlöslichen Wuchsstoff für Hefe, der wahrscheinlich zur Biosgruppe gehört. Bei Zusatz von

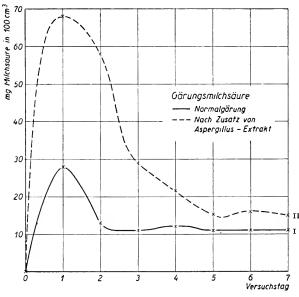


Abb. 2

2% eines derartigen 10% igen, wässrigen Extraktes stieg die Milchsäure nach 5 Tagen bis auf 159 mg in 100 ml Gärlösung an (Kurve II).

Da das p<sub>H</sub> eines Traubenmostes zwischen 3 und 3.5 relativ ungünstig für die Hefe liegt, erstreckt sich diese Angärungsphase bei einer verhältnismässig niedrigen Temperatur zwischen 9 und 12° auf etwa 6 Tage; bei Zimmertemperatur und bei einem für die Hefe günstigen p<sub>H</sub> von 4.8 beschränkt sich diese Phase auf 2 Tage, wie in Laboratoriumsversuchen, z.B. mit einem Moste von Hagebuttenfrüchten, der mit Zucker auf etwa 20% versetzt war, zeigt (Abb. 2). Auch hier ist der Milchsäureanstieg (Kurve I) deutlich zu erkennen und beträgt bei Zusatz von Aspergillusextrakt mehr als das Doppelte (Kurve II).

Auch mit künstlicher Nährlösung, wie sie oben beschrieben wurde, bei einem  $p_H$  von 4.8, ist dieser Anstieg und seine wesentliche Steigerung durch einen Aspergillus-Extrakt als Gärungsaktivator klar ersichtlich, wie die Tabelle I zeigt. Die Angärungsphase dauerte hier ebenfalls nur 2 Tage.

| TABELLE I                              |                                                       |
|----------------------------------------|-------------------------------------------------------|
| mg Milchsäure in 100 ml<br>ohne Zusatz | mgMilchsäure in 100 ml<br>mit Aspergilluszusatz       |
| 6.7                                    | 9                                                     |
| 33.8                                   | 67.5                                                  |
| 22.5                                   | 33.8                                                  |
| 15.0                                   | 16.65                                                 |
|                                        | mg Milchsäure in 100 ml<br>ohne Zusatz  6.7 33.8 22.5 |

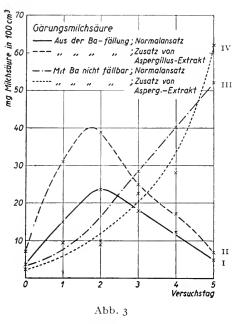
TABELLE I

Die Wirkung des Aspergillusaktivators ist mengenmässig begrenzt und erreicht zwischen 0.2 und 0.5%, auf Pilztrockengewicht berechnet, das Maximum. In einem Gäransatz mit synthetischer Nährlösung wurden nach 17 Stunden die Milchsäurewerte der Tabelle II erhalten. Konzentrationen über 0.5% wirkten hemmend.

TABELLE II

| Ansatz Aspergillus % | mg Milchsäure/100 ml Nährlösung |
|----------------------|---------------------------------|
| 0.05                 | 15.0                            |
| 0.1                  | 27.3                            |
| 0.2                  | 31.6                            |
| 0.5                  | 34.1                            |
| 1.0                  | 21.0                            |

Fraktioniert man die in den beschriebenen Versuchen gebildete Milchsäure derart, dass man mit Bariumacetat und Alkohol bei schwach lackmussaurer Reaktion eine Fällung vornimmt, dann findet man in dieser Bariumfällung nach alkalischer Verseifung ebenfalls Milchsäure. Diese Fällung kann aber keine vorgebildete Milchsäure enthalten, da das Ba-Salz der Milchsäure noch in 75% Alkohol spielend löslich ist. Fällbar mit Barium und Alkohol und zur Milchsäure umgesetzt mit Alkali werden aber von allen Intermediärprodukten bis jetzt nur die Triosephosphorsäuren. Tatsächlich lassen sich auch die annähernden Mengen anorganisches Phosphat nach der Verseifung mit Alkali, herrührend aus einer alkali-empfindlichen Phosphorsäureverbindung, nachweisen. Es ist also sicher anzunehmen, dass in dieser fällbaren Substanz eine Triosephosphorsäure



vorliegt. In Abb. 3 ist ein derartiger Versuch aufgezeichnet. Kurve I ist die gebildete Triosephosphorsäure bei der Angärung ohne Zusatz, Kurve II nach Zusatz von 0.2% getrocknetem Aspergillusmicel. Sie erreicht ihr Maximum bei 40 mg Milchsäure pro 100 ml Ansatz und ist um das 1.75 fache gegenüber dem Normalansatz gesteigert. Beide Werte fallen bei beginnender, sichtbarer Gärung ab und betragen bei voller Gärung, also im stationären Zustand, kaum noch bestimmbare Mengen. Die der Milchsäure entsprechende Phosphat-Menge ist, soweit sie bestimmbar war, in Tabelle III aufgezeichnet.

Die gefundenen Milchsäurewerte in der Mutterlauge der Ba-Fällungen, die der präformierten Milchsäure entsprechen, sind in Kurve III bzw. Kurve IV aufgezeichnet. Aus ihrem Verlauf sieht man deutlich, dass sie im Laufe der Gärung bis zu Werten von ca. 50–60 mg in 100 ml ansteigen. Dieser ungefähre Wert wird

bei allen Gärungen mit lebender Hefe gefunden. Der grösste Teil wird in der Angärungsphase gebildet.

TABELLE III

| Zeit in | mg Milchsäure/100 ml |                 | mg $P_2O_5$ get | funden/100 ml   | ${ m mg~P_2O_5}$ berechnet/100 ml |                 |  |
|---------|----------------------|-----------------|-----------------|-----------------|-----------------------------------|-----------------|--|
| Tagen   | ohne Zusatz          | mit Aspergillus | ohne Zusatz     | mit Aspergillus | ohne Zusatz                       | mit Aspergillus |  |
| I       | 14.8                 | 31.2            | 10.6            | 22.3            | 11.7                              | 24.8            |  |
| 2       | 23.4<br>17.4         | 38.5<br>25.2    | 17.3            | 26.8<br>16.9    | 18.4<br>13.7                      | 30.4<br>19.9    |  |

### ZUSAMMENFASSUNG

Die Angärungsphase mit intakter Hefe zeigt das Ansteigen einer scheinbaren Milchsäurebildung, die durch Zusatz eines wässrigen Aspergillus niger-Extraktes wesentlich gesteigert werden kann. Bei Übergang zur stationären Phase fällt diese Milchsäure bis zu einem während der ganzen Gärung gleichbleibenden Wert ab. Durch Ba-Salzbildung kann man diese Milchsäure in 2 Fraktionen zerlegen, von denen die alkoholunlösliche Ba-Fällung nach ihren Eigenschaften als eine Triosephosphorsäure angesprochen werden muss. Damit scheint bewiesen, dass die Spaltung des Zuckers zu Alkohol auch mit intakter, lebender Hefe ebenso wie beim zellfreien Macerationssaft über Triosephosphorsänre als Zwischenprodukt verläuft. Es ist beliebig unwahrscheinlich, dass die darauffolgenden Reaktionen einen anderen Weg als den in dem Gärungsschema angegebenen einschlagen werden. Die Bildung dieser Triosephosphorsäure ist in der Angärungsphase mit den darauffolgenden Reaktionen nicht synchronisiert, sodass es möglich ist, die vorauseilende Bildung dieser Triosephosphorsäure analytisch zu erfassen. In der stationären Phase jedoch liegt, wie zu erwarten war, dieses Zwischenprodukt nicht angereichert vor.

Weiter zeigen diese Versuche auch den Ursprung der bei jeder Hefegärung entstehenden, geringen Mengen Milchsäure. Er liegt hauptsächlich in der Initialphase und steigt langsam bis zu einem konstant bleibenden Wert während des Gärverlaufes an, der für die untersuchten Weinhefen zwischen

30 und 60 mg/100 ml Most liegt. Es ist noch unklar, ob diese Milchsäure aus einer Änderung des Stoffwechsels von der ruhenden zur sprossenden Hefe stammt, oder ob diese Milchsäure als Produkt der vorauseilenden Triosephosphorsäurebildung aufgefasst werden muss. Im letzteren Fall würde sie natürlich nicht durch alkalische Verseifung entstanden sein. Sie könnte aber aus dem spontanen Zerfall von Triosephosphat zu Methylglyoxal herrühren. Das Methylglyoxal würde dann durch die Methylglyoxalase in Milchsäure umgewandelt. Damit würde zum ersten Mal diesem Enzym in der Hefe eine Funktion zugewiesen (Siehe hierzu auch K. Lohmann<sup>8</sup>).

#### SUMMARY

The initial phase of fermentation with whole yeast shows the onset of an apparent formation of lactic acid, which can be markedly increased by watery extracts of Aspergillus niger. On transition to the stationary phase this lactic acid decreases to an amount which remains constant throughout the fermentation. By forming baryum salts this lactic acid can be separated into two fractions. One of these, the precipitate which is insoluble in alcohol, is to be regarded as a triose-phosphoric acid, according to its properties. This seems to prove that the decomposition of sugar to alcohol by intact living yeast also proceeds by way of triose phosphoric acid as intermediate, just as in the case of cell-free maceration juice. It is rather improbable that the subsequent reactions would follow another route than has been indicated in the scheme of alcoholic fermentation.

The formation of this triose phosphoric acid has not yet been "synchronized" with the following reactions during the initial phase of fermentation, so the preceding formation of this triose phosphoric acid can be demonstrated analytically. In the stationary phase, however, this intermediate is not present in larger amount, as is to be expected.

These experiments also reveal the origin of the small amounts of lactic acid which are formed during each yeast fermentation. This origin is to be found in the initial phase and the amount of lactic acid gradually increases when the fermentation proceeds until a constant value is attained which

is mostly 30-60 mg/100 ml wort for the wine yeasts investigated.

It is not yet clear whether this lactic acid originates from a conversion of the metabolism of resting yeast to that of budding yeast, or whether it must be regarded to be a product of the preceding formation of triose phosphoric acid. In the latter case it would of course not have been formed by alkaline saponification. It could however arise from the spontaneous decomposition of triose phosphate to methylglyoxal. The latter would then be converted into lactic acid by methylglyoxalase. This would be the first time that a function is appointed to this enzyme in the yeast (See also K. Lohmanns).

#### RÉSUMÉ

La phase initiale de la fermentation avec de la levure intacte montre une augmentation de la formation apparente d'acide lactique qui peut être considérablement accrue par l'adjonction d'un extrait aqueux d'Aspergillus niger. Lors du passage à la phase stationnaire la quantité d'acide lactique décroît jusqu'à une valeur qui reste constante pendant toute la durée de la fermentation. Par transformation en sels de barium cet acide lactique apparent peut être séparé en deux fractions; le précipité, de barium insoluble dans l'alcool doit être considéré, d'après ses propriétés, comme provenant d'un acide triose-phosphorique. Ceci semble démontrer que la transformation du sucre en alcool se produit sous l'action de la levure intacte vivante, de même que sous l'action d'un extrait exempt de celluies en passant par l'acide triose-phosphorique comme intermédiaire. Il est assez peu probable que les réactions suivantes passent par un autre chemin que celui indiqué dans le schéma de la fermentation alcoolique.

Dans la phase initiale cette formation d'acide triose-phosphorique n'est pas ''synchronisée' avec les réactions suivantes et c'est pourquoi il est possible de démontrer son existence analytiquement Cependant, ainsi que l'on pouvait s'y attendre, ce produit intermédiaire n'est pas accumulé pendant

la phase stationnaire.

Ces expériences révèlent de plus l'origine des faibles quantités d'acide lactique rencontrées dans toute fermentation produite par la levure. La formation d'acide lactique commence dans la phase initiale at augmente pendant la fermentation jusqu'à une valeur constante qui est de 30 à 60 mg/100

ml de moût pour les levures de vin examinées.

Cet acide lactique provient-il du passage du métabolisme de la levure au repos à celui de la levure bourgeonnante ou bien est-il un produit de la formation précédente d'acide triose-phosphorique? De toutes façons, dans ce dernier cas, il ne pourrait pas provenir d'une saponification alcaline mais bien d'une décomposition spontanée du triose-phosphate en méthylglyoxale. Ce dernier serait ensuite transformé en acide lactique par le méthylglyoxalase. Ce serait la première fois qu'une onction fût attribuée à cet enzyme dans la levure (voir aussi K. LOHMANN<sup>8</sup>).

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# CONFIGURATIONAL RELATIONSHIPS BETWEEN NATURALLY OCCURRING CYCLIC PLANT ACIDS AND GLUCOSE

## TRANSFORMATION OF QUINIC ACID INTO SHIKIMIC ACID

by

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The old idea that meso-inositol could be formed by cyclization of D-glucose gained considerable strength by the determination of the configuration of meso-inositol<sup>1</sup> by Gerda Dangschat, which later was confirmed by Th. Posternak. Similar circumstances could be demonstrated in the field of cyclic plant acids, for instance Quinic Acid and Shikimic Acid. We were able to prove their constitution<sup>2</sup> and their planar configuration<sup>3</sup>.

In 1937 we succeeded<sup>4</sup> by the degradation of shikimic acid into 2-desoxygluconic acid, IX<sup>5</sup> in demonstrating the same configuration for carbon atoms 3, 4 and 5 of shikimic acid as is found for carbon atoms 3, 4 and 5 of D-glucose.

An analogous relationship between quinic acid<sup>6</sup>, which is more commonly found in the plant kingdom, and D-glucose, seemed very probable at that time. This physiologically important relationship could be established with certainty by transforming quinic acid into shikimic acid. In this communication we describe the successful transformation of derivatives of quinic acid into those of shikimic acid<sup>7</sup>.

The use of the acetone compounds of quinic acid, which in previous work with these substances had proven highly satisfactory, met with unexpected difficulties. We therefore employed the formaldehyde derivatives which are described in the preceding paper<sup>8</sup>, after having determined that the methylene group blocked the hydroxyls of carbon atoms 4 and 5 of the quinic acid as did the acetone.

We used a-toluene sulphonyl derivatives of quinic acid and found that the formation of a double bond by the splitting off of the toluene sulphonic acid by alkali only progressed smoothly after conversion to the nitrile, thus considerably weakening the stabilizing influence of the carboxyl group. By prolonged treatment of the 3-acetyl-4,5-formal quinic amide, I<sup>9</sup> with excess of p-toluene sulphonyl chloride and pyridine we performed three reactions in one operation: toluene sulphonylation of the amide, nitrilization of the amide, and finally the splitting off of the toluene sulphonyl group from the nitrile, with the result that the nitrile of the expected 3-acetyl-4,5-methylene shikimic acid, II, could be isolated. This could be converted by means of alkali into the methylene derivative of shikimic acid, III<sup>10</sup> which was transformed into free shikimic acid, V, in acid solution. The identification of shikimic acid was made by melting points, mixed melting points, and optical determinations.

This shows conclusively that quinic acid bears the same steric relationship to D-glucose as that which has already been demonstrated for shikimic acid<sup>11</sup>.

Furthermore, since the structural connection between quinic acid, IV,  $\rightarrow$  shikimic acid, V,  $\rightarrow$  gallic acid, VI, is obvious, it seems to us that in this chemical relationship we have an indication that many hydroaromatic and aromatic plant products are actually formed biologically from carbohydrates. In addition, it might be mentioned that our transformation in vitro of quinic acid to citric acid, VII, by means of periodic acid<sup>12</sup> has perhaps its biological counterpart in the work of Buttenline Suttenline Sutten

#### SUPPLEMENT

Our experiments described in this paper on the transformation of quinic acid into shikimic acid by splitting out water clearly show how strongly the carboxyl of the quinic acid influences its tertiary hydroxyl in the  $\alpha$  position, and probably also the remainder of the molecule.

References p. 203/204.

Our previous papers on the oxidation of quinic acid, shikimic acid and dihydroshikimic acid by means of periodic acid have made available a series of 1.5-dialdehydes, which, depending on their origin, possess either a free or blocked hydroxyl and carboxyl group, or a carboxyl group alone. The possession of these aldehydes led us to an alkaloid synthesis, along the lines of the lobelanine synthesis of Schöpf<sup>14</sup>. The condensation, however, was successful only after the elimination of the electro-negative groups and the choice of a 1.5-dialdehyde which no longer contained any hydroxyl groups and only a carboxyl group in the form of its nifrile. This was the dialdehyde, XI, which is obtained by treating the nitrile of the dihydro-shikimic acid with 2 molecules of periodic acid<sup>2,3,12</sup>.

Experimentally the synthesis was carried out in the following way: Triacetyl dihydro-shikimic acid amide, was transformed into the corresponding nitrile X, by heating with acetic anhydride. The nitrile was de-acetylated with a minimum amount of sodium methylate according to Zemplén, and the free nitrile was transformed into the dialdehyde, XI, by the action of 2 molecules of periodic acid. The dialdehyde was not isolated, but was condensed directly in aqueous solution with 2 molecules of benzoyl acetic acid ester and 1 molecule of monomethylamine at a  $p_H$  of 4.

The 3-cyano-lobelanine, XII, was isolated in a yield of 30% (calculated on the amount of triacetyl dihydro-shikimic acid nitrile), and showed the usual precipitation reaction of alkaloids, e.g. with perchloric acid, picric acid and picrolonic acid. It crystallized in long shining silklike needles similar to those of caffein, and showed a melting point of 143°.

## EXPERIMENTAL

Preparation of the acetyl-methylene-shikimic acid nitrile from monacetyl-methylene-quinic acid amide 5 g monacetyl-methylene-quinic acid<sup>15</sup> were shaken with 10 g ( $2\frac{1}{2}$  molecules) p-toluene sulphonyl chloride in 15 ml dry pyridine for a short time until dissolved. The brown coloured solution was kept for seven days at  $37^{\circ}$ . The solution was then diluted with 20 ml of water, and an oily substance separated. It was allowed to stand with occasional shaking for 15 minutes at room temperature in order to destroy any unused toluene sulphonyl chloride. The solution was then extracted twice with a large volume of chloroform. The united chloroform fractions were next shaken up with small portions of dilute sulphuric acid until all the pyridine was neutralized, and no more acid was used up. The solution was washed with a little water and then dried with sodium sulphate. The mixture was next filtered and the filtrate was evaporated in the vacuum of a water pump to remove all solvent. The light-brown oil (4.5 g) remaining was distilled under high vacuum. A light yellow oil (2.3–2.8 g, i.e., 54–65% of the theoretical yield) distilled over at 0.2 mm and a bath temperature of 150–165°. It had a boiling point of 128°. After a second distillation it was almost colourless, but had a slight odour of toluene sulphonic acid and a minimum content of sulphur.

Preparation of unsaturated nitrile from monacetyl-isopropylidenequinic acid amide16

Reaction and processing follow exactly as described for the corresponding methylene compound. 4.3 g (i.e., 77% of the theoretical yield) unsaturated acetyl-isopropylidene nitrile were obtained from 6.5 g monacetyl-isopropylidene-quinic acid amide. The compound had a light yellow colour and a boiling point of 125°/0.15 mm.

References p. 203/204.

A sample twice redistilled was used for analysis:

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5.068 mg gave 11.195 mg CO_2 and 2.860 mg H_2O; 3.169 mg gave 0.151 ml N_2 (26° and 741 mm). C_{12}H_{15}O_4N (237.1): Calc. C 60.7 H 6.4 N 5.9 Found C 60.8 H 6.3 N 5.4
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Hydrolysis of acetyl-methylene-shikimic acid nitrile to methylene-shikimic acid

3.3 g distilled acetyl-methylene-shikimic acid nitrile were boiled for two and a half hours with 45 ml N sodium hydroxide (about 3 molecules). A condenser was attached to take off the water vapours and the ammonia. Water was added to the distillation flask during boiling so that the volume was not reduced below one-half the original. The condensate was caught in an ice-cooled receiver and at the end of the time the ammonia could be determined almost quantitatively. No formaldehyde was found in the distillate even after acid hydrolysis. The reaction liquid, which was coloured dark brown, was cooled and the alkali was neutralized by addition of 4r ml N sulphuric acid and 4 ml N hydrochloric acid. The weak acetic acid solution was reduced to dryness in the best vacuum obtainable by a water pump, during which time the bath temperature was not allowed to rise above 35°. The residue was extracted thoroughly several times with ethyl acetate, and the united filtered extractions were evaporated under reduced pressure. If crystals are at hand for inoculation, the yellow syrup remaining will begin to crystallize on inoculating. 33–38% of the theoretical yield of crystallized methylene-shikimic acid was obtained from the concentrated ethyl acetate solution, but these crystals still had a yellow colour. Using animal charcoal, a recrystallization from ethyl acetate was made for further purification.

The substance, well crystallized in rhombic plates, had a m.p. of 138° and showed no depression of the melting point on addition of an equal quantity of a preparation made from shikimic acid. The preparation twice recrystallized gave in aqueous solution the following rotation:

$$[a]_{\rm D}^{19^{\circ}} = -88.7^{\circ 17}$$
 (1 dm tube, c = 2.17,  $a_{\rm D}^{19} = -1.93^{\circ}$ ).

Further quantities of the acid could be obtained from the motherliquor of the isolated methyleneshikimic acid in the following manner: The methylene-shikimic acid methyl ester was formed by esterification with diazomethane and was distilled under a high vacuum at a bath temperature of 170–190°. It was then kept for two to three days at 37° together with pyridine and toluene sulphonyl chloride. The toluene sulphonyl-methylene-shikimic acid ester (m.p. and m.p. of the mixture 118–119°) crystallized out readily on gradual addition of water and trituration. This isolated quantity corresponds to a further 15–20% of the theoretical yield of methylene-shikimic acid, so that together about 52% of the acid obtained from the nitrile can be definitely identified as a derivative of the shikimic acid. The methylene-shikimic acid is easily isolated and identified by preparing its toluene sulphonyl-methyl ester, which readily crystallizes. This process is to be recommended, if no inoculation crystals of the free methylene-shikimic acid are at hand or if difficulties appear during the isolation of the free acid. After washing with 50% alcohol the ester is at once obtained in the pure state. For analysis and optical determinations it has to be recrystallized once more from alcohol:

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5071 mg substance gave 10.090 mg CO<sub>2</sub> and 2.350 mg H<sub>2</sub>O 7921 mg substance gave 5170 mg BaSO<sub>4</sub>  \begin{array}{l} \text{C}_{16}\text{H}_{18}\text{O}_{7}\text{S} \text{ (354.2): Calc.} & \text{C}_{54.2} & \text{H}_{5.1} & \text{S}_{9.1} \\ & \text{Found C}_{54.2} & \text{H}_{5.2} & \text{S}_{9.0} \\ \text{[$a$]} & \text{$}^{24^{\circ}}\text{=}-42.5^{\circ} \text{ (in chloroform)}^{18} \text{ (i dm tube, c} = 3.25, $a_{\text{D}}^{24}\text{=}-1.38^{\circ}$).} \end{array}
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Hydrolysis of the unsaturated acetyl-isopropylidene nitrile

The hydrolysis of the unsaturated isopropylidene nitrile can be carried out under the same mild conditions as the corresponding methylene compound. In this reaction the ammonia can also be determined nearly quantitatively after about two hours boiling with dilute alkali. Furthermore, it was found that 25% of the theoretically possible amount of acetone was split off by the alkali. The acetone could be determined in the distillate by titration with alkaline hypoiodite solution and identified as the p-nitro- or dinitrophenylhydrazone. The further processing parallels the procedure used for the methylene nitrile. From the acetonated compound, however, it was not possible to isolate the free acetonated acid, nor to crystallize a derivative of the acetonated unsaturated ester, which had been obtained by esterification with diazomethane and subsequent distillation in a high vacuum. If, however, the unsaturated ester, of which 27% of the theoretical yield was obtained by distillation, is hydrolysed by acetic acid, about 4.5% of the theoretical amount (based on the amount of nitrile used) is obtained in crystallized form 19 as shikimic acid methyl ester. After two recrystallizations from ethyl acetate and ligroin, the m.p. was 112-114% and there was no depression of the melting point when the substance was mixed with equal amounts of a compound prepared from shikimic acid for comparison.

#### SUMMARY

The transformation of quinic acid into shikimic acid by means of the methylene derivatives of these acids has been described. Thus the configuration of the carbon atoms 3,4 and 5 of quinic acid has been shown to be the same as in shikimic acid, which had previously been configurationally related to p-glucose.

3-cyano-lobelanine has been synthesized from dihydroshikimic acid nitrile, benzoyl acetic acid, and monomethył amine under conditions sufficiently mild so that they might exist in plant or animal organisms.

#### RÉSUMÉ

Nous avons décrit la transformation de l'acide quinique en acide shikimique à l'aide des dérivés méthyléniques de ces acides. Nous avons montré ainsi que la configuration des atomes de carbone 3, 4 et 5 dans l'acide quinique est la même que dans l'acide shikimique, dont la configuration avait été précédemment reliée à celle du p-glucose.

La 3-cyano-lobélanine a été synthétisée à partir du nitrile de l'acide dihydro-shikimique, de l'acide benzoylacétique et de la monométhylamine sous des conditions suffisamment douces pour exister dans l'organisme végétal ou animal.

#### ZUSAMMENFASSUNG

Wir beschreiben die Umwandlung der Chinasäure in die Shikimasäure über die entsprechenden Methylenderivate. Es wurde also gezeigt, dass die Konfiguration der Kohlenstoffatome 3, 4 und 5 in der Chinasäure dieselbe ist wie in der Shikimasäure, deren Konfiguration schon früher auf die der D-Glucose zurückgeführt wurde.

3-Cyanolobelanin wurde aus Dihydroshikimisäure-nitril, Benzoylessigsäure und Monomethylamin unter milden Bedingungen synthetisiert, wie sie auch im pflanzlichen oder tierischen Organismus vorkommen können.

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18 After acid hydrolysis following the prescription given in the "9th Communication on Quinic Acid and derivatives" free shikimic acid is obtained:

its m.p. and m.p. of a 50% mixture 184–185°; 
$$[a]_{\rm D}^{19}$$
° =  $-183$ ° (in water, 1 dm tube, c = 1.23,  $a_{\rm D}^{19}$  =  $-2.25$ °).

19 The small yield of *crystallized* substance suggests that the acetyl-isopropylidene-shikimic acid nitrile contains, unlike the corresponding methylene compound, a considerable quantity of a 1,2 unsaturated product.

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# PARTIAL PURIFICATION OF ISOCITRIC DEHYDROGENASE AND OXALOSUCCINIC CARBOXYLASE\*

by

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It has been shown<sup>1,2</sup> that the over-all reversible Reaction 1, catalysed by enzymes present in a number of tissues, involves two steps (Reactions 2 and 3).

1. 
$$d$$
-Isocitrate + TPN $_{ox} \stackrel{Mn^{++}}{\rightleftharpoons} \alpha$ -ketoglutarate + CO $_2$  + TPN $_{red}$ 

2. 
$$d$$
-Isocitrate + TPN<sub>ox</sub>  $\Longrightarrow$  oxalosuccinate + TPN<sub>red</sub>

3. Oxalosuccinate 
$$\stackrel{Mn++}{=}$$
  $\alpha$ -ketoglutarate  $+$  CO<sub>2</sub>

Crude enzyme solutions from heart muscle<sup>1</sup>, liver<sup>3</sup> and higher plants<sup>4</sup> catalyse Reaction 1 in either direction, as well as the decarboxylation of oxalosuccinate (Reaction 3), in the presence of added manganous ions. Reaction 2 can be shown to occur in either direction with the same enzyme solutions when  $Mn^{++}$  is excluded<sup>1</sup>.

Partial purification of isocitric dehydrogenase, as tested by Reaction I, was previously reported<sup>1</sup>. A four-fold purification of the activity exhibited by extracts of acetone-dried pig heart, with very low yield, was obtained at that time. Lynen and Scheres have recently reported the synthesis of oxalosuccinic acid and the catalysis of the decarboxylation of this compound by enzymes from various sources. Their work, carried out without knowledge of the work of this laboratory, led essentially to the same results. They also reported partial purification of the oxalosuccinic carboxylase activity (Reaction 3) of horse liver.

A somewhat improved method of purification of the isocitric dehydrogenase and oxalosuccinic carboxylase activities of pig heart, as determined according to Reactions 1 and 3, is described in this paper. A six-fold purification of the activity of the extracts with a yield of about 15% has been obtained. There was no separation of activities as tested by Reactions 1 and 3, but both these activities were increased with respect to malic dehydrogenase. Thus, the question whether Reactions 2 and 3 are catalysed by distinct enzymes (isocitric dehydrogenase and oxalosuccinic carboxylase respectively), or by a single enzyme, still remains unsettled.

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#### OPTICAL TESTS AND ENZYME UNITS

Over-all Reaction. — The activity determination is based on Reaction 1. The early rate of reduction of triphosphopyridine nucleotide (TPN) in the presence of enzyme,  $Mn^{++}$ , and an excess of isocitric acid, is proportional to the concentration of the enzyme within certain limits. The measurement is carried out in the Beckman spectrophotometer at wave-length 340 m $\mu$  using either glass or silica cells of 1.0 cm light path. One enzyme unit was defined as the amount of enzyme causing an increase in optical density of 0.01 per minute calculated for the third 15 second period after the start of the reaction.

The reaction mixture, in a final volume of 3.0 ml contained 0.025 M glycyl-glycine buffer  $p_H$  7.4, 0.6·10<sup>-3</sup> M  $MnCl_2$ , 0.45·10<sup>-4</sup> M  $TPN_{ox}$ , enzyme, and 0.175·10<sup>-3</sup> M d,l-isocitrate. The volume was made up with water adjusted to a temperature of  $22-23^{\circ}$ . The blank cell, for setting at 100% light transmission contained all the above components except TPN. The reaction was started, after taking a zero time reading, by addition of either enzyme or isocitrate. The presence of phosphate in concentrations higher than 0.0003 M should be avoided because turbidity, due to precipitation of manganous phosphate, may develop. Typical results obtained with an extract of washed acetone-dried pig heart containing 6.0 mg of protein per ml are shown in Table I.

TABLE I
OPTICAL TEST FOR REACTION 1
PROPORTIONALITY OF RATE TO ENZYME CONCENTRATION

| Enzyme<br>concentration |         | Units | Specific activity |
|-------------------------|---------|-------|-------------------|
| mg protein in 3.0 ml    |         |       | units mg protein  |
| 0.012                   | + 0.005 | 2.0   | 166               |
| 0.024                   | + 0.012 | 4.8   | 198               |
| 0.036                   | + 0.017 | 6.8   | 188               |
| 0.048                   | + 0.023 | 9.2   | 192               |
| 0.060                   | + 0.026 | 10.4  | 173               |
|                         |         |       | Average 183       |

The protein content of the enzyme solutions was determined spectrophotometrically by measuring the absorption of light at wave-lengths 280 and 260 m $\mu$ . The protein concentration was calculated from the absorption at 280 m $\mu$  with a correction for the nucleic acid content from the data given by Warburg and Christian<sup>6</sup>.

Oxalosuccinic Carboxylass. — The oxalosuccinic carboxylase activity (Reaction 3) was determined by means of a rapid and sensitive optical test. The test is based on the fact that, in the presence of  $Mn^{++}$  and oxalosuccinate, the enzyme causes a pronounced increase in the absorption of light at the wave-length 240 m $\mu$ , presumably as a result of increased formation of an intermediate oxalosuccinate-manganese complex; this increase is followed by a rapid drop indicating decarboxylation. The early rate of increase of light absorption is, within certain limits, proportional to the concentration of enzyme. The measurement is carried out in the Beckman spectrophotometer using silica cells of 1.0 cm light path. One enzyme unit was defined as the amount of enzyme causing an increase in optical density of 0.01 per minute calculated for the first 15 second period after the start of the reaction.

The reaction mixture, in a final volume of 3.0 ml, contained 0.134 M potassium chloride, enzyme, 0.167·10<sup>-3</sup> M MnCl<sub>2</sub>, and approximately 0.167·10<sup>-3</sup> M oxalosuccinate\*. The volume was made up with water adjusted to a temperature of 15°. The blank cell contained no oxalosuccinate. The reaction was started by addition of oxalosuccinate, which was blown into the mixture from a Lang-Levy micropipette<sup>9</sup>, and readings of the optical density were made at 15 second intervals thereafter for 1 or 2 minutes. The optical density of the oxalosuccinate was determined separately and furnished the zero time value. The amount of enzyme was so adjusted that an increase in optical density not below 0.07 nor above 0.20 was obtained in the first 15 seconds. The reason for the presence of potassium chloride is that it was found to increase the activity of the enzyme. This effect appears to be a non-specific one caused by the increased ionic strength? The presence of phosphate in concentrations higher than 0.0003 M should be avoided for the reasons already stated. Typical results obtained with the acetone powder extract of pig heart are shown in Fig. 1.

<sup>\*</sup> Prepared as previously described8.

Malic Dehydrogenase. — The optical test for malic dehydrogenase activity is based on Reaction 4.

(4) Oxalacetate + DPN<sub>red</sub>  $\rightleftharpoons l$ -malate + DPN<sub>ox</sub>

The test is carried out in the Beckman spectrophotometer, at wave-length 3.40 m $\mu$ , using cells of 1.0 cm light path. It is based on the fact that the early rate of oxidation of reduced diphosphopyridine nucleotide (DPN<sub>red</sub>) by oxalacetate is proportional to the enzyme concentration within certain limits. One enzyme unit was defined as the amount of enzyme causing a decrease in optical density of 0.01 per minute calculated for the third 15 second period after the start of the reaction. The reaction mixture, in a final volume of 3.0 ml, contained 0.025 M glycylglycine buffer pH 7.4, 0.4·10<sup>-4</sup> M DPN<sub>red</sub>, enzyme, and 0.25·10<sup>-3</sup> M oxalacetate. The volume was made up with water adjusted to a temperature of 22–23°. The blank cell contained no DPN. The reaction was started, after taking a zero time reading of the optical density, by addition of either oxalacetate or enzyme.

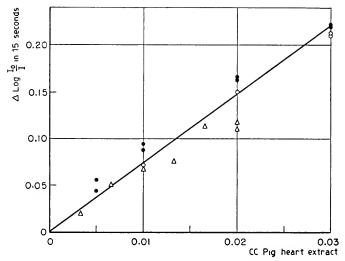


Fig. 1. Optical test for oxalosuccinic carboxylase (Reaction 3). Proportionality of rate to enzyme concentration.

#### PREPARATION OF ENZYME

Extraction. — Acetone-dried pig heart was prepared by the method described by Straub<sup>10</sup>. The dry material was ground to a fine powder in a mechanical mortar. The powder was extracted with 0.1 M phosphate buffer  $p_{\rm H}$  7.4 at room temperature following the method of Straub<sup>10</sup>.

Ammonium Sulphate Fractionation. — The clear extract was cooled to  $\circ$ °, brought to 50% saturation with solid ammonium sulphate, and the mixture was filtered with suction in the cold room using filter-aid (Hyflo-Supercel) to facilitate filtration. The precipitate was discarded and the supernatant was brought to 60% saturation with solid ammonium sulphate. The mixture was filtered as before. The supernatant was discarded and the precipitate was dissolved in cold 0.04 M phosphate buffer  $p_H$  7.4 to give a concentration of about 3% protein. The solution was clarified by filtration and dialysed against 0.04 M phosphate buffer  $p_H$  7.4 at  $2-3^\circ$  for 4-5 hours.

Ethanol Fractionation. — The dialysed solution was fractionated with ethanol at low temperature. Details of the procedure have been described elsewhere 11. The most active fraction was usually obtained between 20 and 30% ethanol by volume at  $-5^{\circ}$ . The precipitate was collected by centrifugation at  $-5^{\circ}$ , dissolved in cold o.or M phosphate buffer  $p_{\rm H}$  7.4, and dialysed for a few hours at  $2-3^{\circ}$  against the same buffer.

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TABLE II

PARTIAL PURIFICATION OF ISOCITRIC DEHYDROGENASE AND OXALOSUCCINIC CARBOXYLASE

Soo gm of powder of Washed, acetone-dried, pig heart

| Step                                                                        | Volume<br>of so- | Protein | Oxalosu<br>carbox |       | Isocit<br>dehydrog | enase*  | Ratio          | Yield<br>(OS car- | Malic de<br>drogen |     |
|-----------------------------------------------------------------------------|------------------|---------|-------------------|-------|--------------------|---------|----------------|-------------------|--------------------|-----|
| lution<br>ml                                                                | mg               | Units   | S.A.**<br>(a)     | Units | S.A.**<br>(b)      | (a)/(b) | boxylase)<br>% | Units             | S.A.**             |     |
| Extract<br>(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub><br>fractionation | 8 300            | 48200   | 22244000          | 462   | 7968000            | 165     | 2.8            | 100               | 17928000           | 373 |
| (0.5–0.6 sat.)<br>Ethanol                                                   | 134              | 7210    | 12542400          | 1740  |                    |         |                | 55                |                    |     |
| fractionation $(20-25\%)$                                                   | 37               | 1 254   | 3 596 400         | 2860  | 1108150            | 885     | 3.2            | 16                | 1116956            | 890 |

 $<sup>^{\</sup>star}$  Over-all reaction isocitrate + TPN<sub>ox</sub>  $\rightleftharpoons a$ -ketoglutarate + CO<sub>2</sub> + TPN<sub>red</sub>

\*\* Specific activity (units/mg protein)

These preparations are very unstable and lose activity rather rapidly even when stored at 0°. If dried from the frozen state, 30 to 40% of the activity is lost but, on the other hand, the remaining activity persists unchanged for many months when the dry powder is stored in the cold over calcium chloride. The preparations contain no aconitase and only traces of lactic dehydrogenase.

The results of a typical fractionation are summarized in Table II.

Occasionally the purification obtained after ammonium sulphate and ethanol fractionation may be lower than that reported in Table II. The purity of these preparations can be increased about 1.5 times, with a yield of 60% or better, by adsorption on calcium phosphate gel. For this purpose the enzyme solution is diluted with 0.01 M phosphate buffer p<sub>H</sub> 7.4 to give a protein concentration of about 1%. The adsorption is carried out successively with small amounts of the gel, until all the activity has been removed from solution, and the sediments are separately eluted with 0.1 M phosphate buffer p<sub>H</sub> 7.4. The eluates are tested separately and the best ones are combined. The calcium phosphate gel was prepared following the directions of Keilin and Hartree<sup>12</sup>.

## COMPARISON OF MANOMETRIC AND OPTICAL DETERMINATION OF OXALOSUCCINIC CARBOXYLASE ACTIVITY

The specific oxalosuccinic carboxylase activity of the extract of acetone-dried pig heart, as determined manometrically, has been previously reported<sup>2</sup>. The determinations were carried out at  $p_H$  5.6 and 15°, in the presence of 0.0014 M MnCl<sub>2</sub> and 0.0065 M oxalosuccinate, and the CO<sub>2</sub> evolution due to spontaneous decarboxylation was subtracted from the total to obtain the enzyme-catalysed decarboxylation rate. Pig heart extract catalysed the evolution of 70  $\mu$ l of CO<sub>2</sub> during the first 5 minutes per mg of protein. The activity of pig liver extract was about one tenth of this value.

The manometric specific activity of 70 corresponds to an optical specific activity of 462 (cf. Table II). Thus, the activity of the ethanol fraction of Table II is 2,860  $\cdot$  70/462 or 435  $\mu$ l of CO<sub>2</sub> in 5 minutes per mg of protein (at 15°). The best fraction of Lynen and Scherer<sup>5</sup> had a specific activity of 100  $\mu$ l CO<sub>2</sub> (corrected for spontaneous decarboxy-

lation) in the first 2 minutes per mg of protein, tested at 30°\*. Allowing for the difference in temperature in the manometric tests of the two laboratories, it would appear that the specific oxalosuccinic carboxylase activity of Lynen and Scherer's preparation from horse liver was only about one fourth of that obtained by us starting with pig heart.

#### INHIBITION OF OXALOSUCCINIC CARBOXYLASE BY ISOCITRIC ACID

It has been reported that isocitric acid strongly inhibits the enzymatic decarboxylation of oxalosuccinic acid as followed manometrically<sup>2</sup>. As shown in Fig. 2, this inhibition can also be observed under the conditions of the optical test. The test system

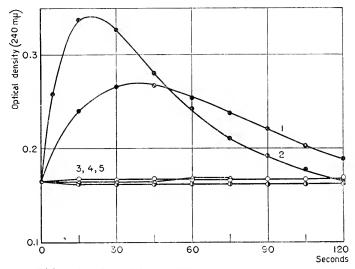


Fig. 2. Inhibition of oxalosuccinic carboxylase activity by isocitric acid; optical test. (Description in text).

was as indicated in a previous section. Curves 1 and 2 were obtained with 0.02 and 0.04 ml respectively of the acetone powder extract of pig heart (about 0.12 and 0.24 mg of protein). Oxalosuccinate (final concentration, 0.167 · 10<sup>-3</sup> M) was added at zero time in all cases. Curves 3 (-0-0-) and 4 ( $-\Delta-\Delta$ ) both with 0.04 ml of extract and either 0.35 · 10<sup>-3</sup> M (curve 3) or 0.35 · 10<sup>-4</sup> (curve 4) d,l-isocitrate. Curve 5 ( $-\Phi-\Phi$ ) with 0.02 ml of extract and 0.35 · 10<sup>-3</sup> M d,l-isocitrate.

#### Acknowledgement

We are indebted to Mr Morton C. Schneider for technical assistance.

#### SUMMARY

Partial purification of the isocitric dehydrogenase and oxalosuccinic carboxylase activities of pig heart has been obtained by means of ammonium sulphate and ethanol fractionation of an acetone

<sup>\*</sup> Manometric test with 0.001 M MnSO<sub>4</sub> and 0.002 oxalosuccinate, p<sub>H</sub> 6.0. The purification procedure involved water extraction of the fresh liver, precipitation with acetone, fractionation with nucleic acid between p<sub>H</sub> 5.18 and 4.6, and precipitation with ethanol. The average specific activity of solutions of the acetone precipitate was 3.8. Yields were not reported and the fractions were not tested for isocitric dehydrogenase.

powder extract. The purification reached was about six-fold with a yield of about 15%. No separation of the two activities has thus far been accomplished. The strong inhibition of oxalosuccinic carboxylase activity by isocitric acid has been confirmed using an optical test system.

#### RÉSUMÉ

Nous avons réussi une purification partielle des principes actifs de l'isocitrate-déhydrogénase et de l'oxalosuccinate-carboxylase par fractionnement au sulfate d'ammonium et à l'éthanol d'un extrait acétonique de poudre de coeurs de Pigeon. Après purification l'activité était environ six fois plus grande, tandisque le rendement était de 15% environ. Les deux activités n'ont pas encore pu être séparées. Nous avons confirme par test optique que l'activité de l'oxalosuccinate-carboxylase est fortement inhibée par l'acide isocitrique.

#### ZUSAMMENFASSUNG

Die Isocitrat-Dehydrogenase und die Oxalosuccinat-Carboxylase aus einem Acetonextrakt von getrocknetem pulverisierten Taubenherz wurden durch fraktionierte Fällung mit Ammoniumsulphat und Äthanol teilweise gereinigt. Die Aktivität wurde ungefähr sechsmal angereichert, wobei die Ausbeute etwa 15% betrug. Es wurde keinerlei Trennung der beiden Aktivitäten beobachtet. Die starke Hemmung der Oxalosuccinat-Carboxylase durch Isozitronensäure wurde durch einen optischen Test bestätigt.

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## SPECTROPHOTOMETRIC MEASUREMENTS OF THE ENZYMATIC FORMATION OF FUMARIC AND CIS-ACONITIC ACIDS

by

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Fumaric and cis-aconitic acids are intermediates in the main pathway of substances oxidized through the tricarboxylic acid cycle. With the exception of the keto-acid oxidases, the enzymes participating in the cycle have been obtained in solution and after purification can be studied in isolated and defined systems. Compounds such as fumaric and cis-aconitic acid with an unsaturated C=C linkage have a marked absorption in the ultraviolet. This property can be utilized in a spectrophotometric test, measuring appearance and disappearance of these substances in the course of enzymatic reactions.

A rapid and convenient test for the enzymes catalysing the formation of fumaric acid from malic acid or aspartic acid and the formation of *cis*-aconitic acid from citric acid or isocitric acid will be described in this paper.

#### EXPERIMENTAL

Ultraviolet Absorption Spectrum of Fumaric Acid and Cis-Aconitic Acid

The ultraviolet absorption spectrum of the sodium salts of these two acids is recorded in Fig. 1. The fumaric acid used in this experiment was a recrystallized commercial preparation; the cis-aconitic acid was kindly supplied by Dr S. Ochoa. As can be seen from Fig. 1, the absorption of these compounds shows a steady rise toward the short wave lengths. Because proteins and nucleic acids absorb considerable amounts of ultraviolet light in this region, enzymes used for spectrophotometric studies must have a fairly high turnover number so that activity measurements can be

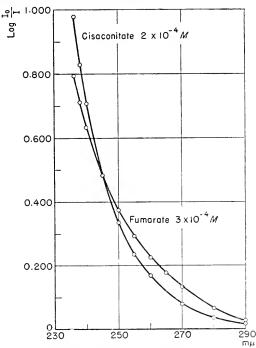


Fig. 1. Ultraviolet absorption spectrum of sodium fumarate and sodium cis-aconitate.

carried out at high enzyme dilutions. The activity of enzymes with a low turnover number can be tested spectrophotometrically only after considerable purification, with

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removal of interfering absorbing substances, particularly proteins and nucleic acid.

Of the enzymes catalyzing the formation of unsaturated intermediates of metabolism, fumarase, aconitase and aspartase were selected for study.

#### PREPARATION OF ENZYMES

a) Fumarase. Fumarase was prepared according to the method of Laki and Laki and fumarase activity was measured at each stage of the purification<sup>2</sup>. It was found that the preparation at the final stage still contained contaminating proteins. The crystalline precipitate obtained was found to have lost most of the fumarase activity after four subsequent recrystallizations while the supernatant retained the fumarase activity<sup>2</sup>. These findings confirm the report by Scott<sup>3</sup> who observed that the crystalline fraction lost fumarase activity on recrystallization while the amorphous fraction had a specific activity equal to that ascribed to the crystals by Laki and Laki. Furthermore, the purified fumarase preparations of Laki and Laki still contain considerable quantities of contaminating proteins. Appreciable aconitase activity has been found in these preparations as will be described below, as well as very active lactic acid dehydrogenase which represents about 20% of the protein present<sup>2</sup>.

b) Aconitase. Fumarase prepared by the method of Laki and Laki, and kindly supplied by Dr J. B. V. Salles, was found to contain an active aconitase as noted above. This preparation of fumarase had been kept at o° for several weeks and retained considerable aconitase activity. Because of the known lability of purified aconitase, it was decided to investigate this preparation further.

Fumarase was prepared, therefore, according to the method of Laki and Laki and fumarase and aconitase activity were measured in all fractions<sup>2</sup>. A large proportion of the aconitase activity was retained by the heart muscle pulp after thorough washing with water; the pulp was then extracted by the phosphate buffer treatment used for obtaining the fumarase activity <sup>1</sup>. Both aconitase and fumarase were purified. Aconitase showed a somewhat greater sensitivity to the acid ph used in the course of the purification. On fractionation with ammonium sulphate, the fumarase precipitated at lower salt concentrations, so that partial separation of the two enzymes was accomplished.

An aconitase preparation was also made from Fleischmann's baker's yeast. Maceration juice was obtained by extracting dried yeast with M/15 disodium phosphate for 3 hours at 37°. The maceration juice was fractionated at -5° with acetone. An active fraction was obtained which precipitated between 30 and 50% acetone concentration. This was dissolved in cold water and dialysed for two hours against running tap water. Following centrifugation, the supernatant was further fractionated by the addition of solid ammonium sulphate. The precipitate obtained at 50% saturation was collected. Solid ammonium sulphate was added to the supernatant and the fractions precipitated up to 80% saturation were also collected. The aconitase activity of these fractions will be described later in this paper.

c) Aspartase. This enzyme was prepared from E. coli (strain B). The bacteria were grown in neopeptone broth for 18 hours at 37° with vigorous aeration, then centrifuged and washed once distilled water. They were then suspended in a small volume of distilled water and disintegrated by sonic vibration for five minutes. After centrifugation for 20 minutes at 18000 rpm in a refrigerated centrifuge, the supernatant was fractionated by means of ammonium sulphate. The precipitate obtained at 50% saturation was dissolved and dialysed against distilled water at 0° for 24 hours. This preparation of aspartase was used for the studies described in this paper and was found to be quite stable if kept at 0°.

#### SPECTROPHOTOMETRIC MEASUREMENTS

a) Fumarase. The enzymatic activity of fumarase was determined in a Beckman DU quartz spectrophotometer. The final volume was 3 ml including 0.05 M potassium-phosphate buffer at  $p_H$  7.4 and 0.05 M sodium L-malate. After addition of the enzyme, the changes in absorption at 240 m $\mu$  were recorded at intervals of 15 seconds. The control cell contained all the solutions except the substrate. The enzymatic reaction follows a zero order course for several minutes and is measured during this period. One unit is defined as a change of  $\log \frac{I_0}{I}$  of 0.001 per minute. The increments in optical density at 240 m $\mu$  are proportional to the amount of enzyme added (Fig. 2).

<sup>\*</sup> Sonic oscillator manufactured by Ratheon Corp., Waltham, Massachusetts, U.S.A.

Under these experimental conditions the Michaelis constant for fumarase as determined by the method of Lineweaver and Burk<sup>4</sup> was  $4.1 \cdot 10^{-3}$  (moles  $\times$  liter<sup>-1</sup>) with sodium L-malate as substrate.

The enzymatic activity of fumarase can also be followed with sodium fumarate as the substrate. Due to the high specific absorption of fumaric acid, only limited amounts of this substrate, which are not sufficient to saturate the enzyme, can be used in the spectrophotometric test. The rates, therefore, are slower and fall off more rapidly than with L-malic acid as the substrate. However, with an active enzyme preparation the

equilibrium is quite rapidly established from either direction.

b) Aconitase. For the measurement of aconitase activity the test system was the same as that for fumarase except that the substrate used was either 0.03 M sodium citrate or 0.01 M sodium d.L-isocitrate (kindly supplied by Dr S. Ochoa). Since the enzyme is unstable in dilute solutions, all estimations were carried out immediately following dilution in 0.1 M phosphate buffer. The enzymatic activity followed a zero order course for several minutes and was proportional to the amount of enzyme added (Fig. 2).

The specific activity (units/mg protein) of aconitase preparations when tested with isocitrate was always found to be greater than with citrate. Considerable variation in the relative activities was found in different fractions during purification. Although no evidence was obtained of a separation of the enzyme activity for the two substrates, the respective activities, for the sake of convenience, are referred to as citrase and isocitrase. Thus, in a crude heart extract, a ratio isocitrase/citrase activity of 2.1 was found,

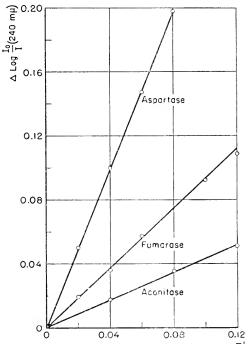


Fig. 2. Quantitative determination of fumarase, aconitase and aspartase. Relation of enzyme concentration to activity per minute.

while the purified preparation<sup>2</sup> had a ratio of 7.5. Similarly, the fractions obtained from yeast by acetone and ammonium sulphate precipitation, showed considerable variation in the relative citrase and isocitrase activities. The ammonium sulphate precipitate obtained at 50% saturation showed an isocitrase/citrase ratio of 2.0, while the fractions obtained between 60 and 80% saturation showed a ratio of about 7.0.

The Michaelis constant of aconitase measured with sodium citrate as substrate was found to be 1.1·10<sup>-3</sup> and for D-isocitrate  $4\cdot10^{-4}$  M.

c) Aspartase. This enzyme was measured in the same manner as the other hydrases with 0.15 M sodium aspartase as the substrate. A high concentration of substrate is required for maximal activity of this enzyme. With substrate concentration sufficient for enzyme saturation, proportionality between enzyme concentration and increments in optical density was found (Fig. 2).

The Michaelis constant of aspartase was found to be in the neighbourhood of References p. 214.

 $3 \cdot 10^{-2}$  M. Some variation around this value was found with different preparations. This variation might be explained by the presence of two different aspartases reported by  $GALE^5$ .

#### DISCUSSION

Rapid and convenient spectrophotometric methods for the determination of glycolytic enzymes of the Meyerhof-Embden scheme have been developed by Warburg and his school. These methods have been valuable in following purification and also for kinetic studies of these enzymes. The high absorption coefficients in the ultraviolet of unsaturated compounds such as fumaric and aconitic acid have been made the basis for a method of measuring their enzymatic formation. Other compounds such as crotonic and vinyl-acetic acid were also found to show a high absorption in the ultraviolet. These latter compounds are known to be metabolized by animal tissues and by bacteria and may be intermediates of fatty acid metabolism. In view of their high specific light absorption, their enzymatic formation and breakdown could be followed by spectrophotometric tests similar to those described in this paper.

The occurrence of unsaturated compounds as intermediates of metabolism of amino acids such as serine and threonine has been postulated. The probably high absorption in the ultraviolet of such intermediates may help in the elucidation of the pathway of the metabolic breakdown of these amino acids. Advantage has been taken of the high absorption coefficients of reduced coenzymes I and II, keto acids, dehydropeptides, and amino acids, such as tyrosine for enzymatic studies with these compounds. The present study shows that the metabolism of unsaturated organic substances may be followed by a similar technique.

#### SUMMARY

A spectrophotometric method of measuring the enzymatic formation and disappearance of umaric and cis-aconitic acids is reported.

#### RÉSUMÉ

Nous décrivons une methode spectrophotométrique qui permet de mesurer la formation et la disparation enzymatique de l'acide fumarique et de l'acide cis-aconitique.

#### ZUSAMMENFASSUNG

Eine spektrophotometrische Methode zur Messung der enzymatischen Bildung und Zerstörung von Fumarsäure und cis-Akonitsäure wird beschrieben.

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## THE INTERCONVERSION OF THE RETINENES AND VITAMINS A IN VITRO

by

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In the summer of 1933 I was working as a National Research Council Fellow in Otto Meyerhof's Institute in Heidelberg, measuring the distribution of phosphates in the frog retina in light and darkness. I had noticed that the trichloracetic acid used to extract the phosphates turned the red colour of the dark adapted retina to bright orange, and that thereafter the retina behaved as a  $p_H$  indicator, orange in acid and colourless in alkaline solution. Light adapted retinas were colourless under all circumstances.

All about us the Third Reich was coming into flower, and the laboratory remained an island of sanity in a world increasingly committed to unreason and repression. Under the urging of the Society of Animal Friends, led by a retired general, the government of Baden had forbidden the killing of frogs — that is, German frogs; there seemed to be no objection to importing foreign frogs for laboratory use.

In August, just after Professor Meyerhof and his assistants left on their vacations, and I had all but terminated my phosphate experiments, a large shipment of frogs arrived from Hungary. The *Diener* was prepared to throw them into the Neckar, but it seemed a pity to waste them, and I decided to use them to try to learn something of the orange  $p_H$  indicator which results from the destruction of rhodopsin in the retina. It was under these circumstances that I found retinene<sub>1</sub>, and had a first view of its interplay with vitamin  $A_1$  in the rhodopsin cycle.

It is only within the past few months that the chemistry of these relationships has been clarified. At a key point in this investigation it fell in with the pattern of Meyerhof's classic experiments on the role of cozymase in the lactic fermentation. For cozymase is also the substance which reduces the retinenes to the vitamins A; and to learn this we entered on a line of experiment developed by Meyerhof many years before.

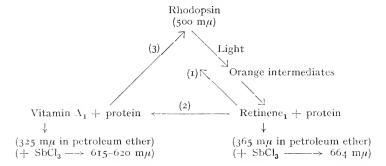
It is therefore in a double sense that I offer this essay to Otto Meyerhof: first, for his personal connection with its beginnings; and again, for the debt to him and to his work which I share with all who do biochemistry.

#### RETINENE, AND VITAMIN A

Vision in dim light is mediated in all vertebrates through the retinal receptors known as rods. In land and sea vertebrates, these organs contain the red, light-sensitive

<sup>\*</sup> The recent investigations described in this paper have been supported in part by the Medical Sciences Division of the Office of Naval Research.

pigment rhodopsin. This substance takes part with the carotenoids retinene<sub>1</sub> and vitamin  $A_1$  in a cycle of reactions of the following form\*:



Rhodopsin bleaches in the light over unstable orange intermediates to a mixture of yellow retinene<sub>1</sub> and colourless protein; the retinene<sub>1</sub> is then transformed to colourless vitamin  $A_1$ ; and both vitamin  $A_1$  and retinene<sub>1</sub>—or its orange precursors—recombine with protein to form new rhodopsin (Wald, 1935–36 a, b).

One has only to separate the retina from contact with the underlying tissues which line the optic cup to abolish the synthesis of rhodopsin from vitamin A<sub>1</sub> (reaction 3). According to KÜHNE this process requires the cooperation of a living pigment epithelium (EWALD AND KÜHNE, 1878, page 255; KÜHNE, 1879).

When the system is further reduced by bringing rhodopsin into aqueous solution, processes (1) and (2) are usually also eliminated. Nothing then remains but the succession of light and "dark" reactions which transform rhodopsin into retinene, and protein.

The present paper is concerned primarily with reaction (2), the conversion of retinene<sub>1</sub> to vitamin  $A_1$ . This is a slow, irreversible process which goes to completion in the isolated retina in about an hour at room temperature<sup>\*\*</sup>. In 1942–43 we succeeded in bringing this process into a cell-free brei prepared from cattle retinas; and recently BLISS (1948) has shown that it occurs under certain conditions in freshly prepared rhodopsin solutions. These demonstrations that it can proceed *in vitro* form a prelude to the present experiments. Their other antecedent is the clarification of chemical relations between retinene<sub>1</sub> and vitamin  $A_1$ , due primarily to the work of Morton and his colleagues in Liverpool.

Vitamin  $\Lambda_1$  is the primary alcohol  $C_{19}H_{27}CH_2OH$ . Ball, Goodwin, and Morton (1948) found that on mild oxidation this is transformed to a product which agrees in spectrum and antimony chloride reaction with retinene<sub>1</sub>. They have crystallized this product and shown it to be an aldehyde, which they believe to be simply vitamin  $\Lambda_1$  aldehyde,  $C_{19}H_{27}CHO$ . Their analytic data do not establish this formulation unequivocally as yet; but all that is now known of retinene<sub>1</sub> from the work of Morton's laboratory and our own is consistent with the view that it is vitamin  $\Lambda_1$  aldehyde. We shall accept this as its structure in what follows.

<sup>\*</sup> The wavelength values written below components of this cycle represent maxima in the absorption spectra of these substances in solution, or, when so indicated, of the products which these substances yield when treated with antimony trichloride.

Designating this as an irreversible process is not intended to exclude the possibility that it is in fact reversible, but with the equilibrium far over toward vitamin A formation. It might for example be possible by greatly increasing the concentration of vitamin A<sub>1</sub> in the system to demonstrate a small reversion to retinene<sub>1</sub>.

#### THE OXIDATION OF VITAMIN A<sub>1</sub> TO RETINENE<sub>1</sub>

In their simplest procedure for oxidizing vitamin  $A_1$  to retinene<sub>1</sub>, Ball *et al.* (1946) added a little manganese dioxide powder to a solution of vitamin  $A_1$  in petroleum ether, and placed this mixture in a refrigerator. After 3-4 days they found that retinene<sub>1</sub> had replaced vitamin  $A_1$  in the supernatant solution.

On examining this process we found its mechanism to be as follows. Vitamin  $\Lambda_1$  is strongly adsorbed on manganese dioxide, and is oxidized to retinene<sub>1</sub> in the adsorbed condition. Retinene<sub>1</sub> is much less strongly adsorbed and so is displaced from the manganese dioxide by new vitamin  $\Lambda_1$  as fast as it is formed. In this way all the vitamin  $\Lambda_1$  passes over the manganese dioxide surface, and is replaced by retinene<sub>1</sub> in the supernatant solution. At the close of the process, the final charge of vitamin  $\Lambda_1$  on the adsorbent is oxidized to retinene<sub>1</sub>, and then, with no vitamin  $\Lambda_1$  remaining to displace it, is oxidized further to what I have called the 545 m $\mu$ -chromogen. This can be recovered from the manganese dioxide by elution with a polar organic solvent such as ethanol.

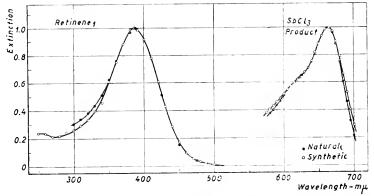


Fig. 1. Comparison of natural and synthetic retinene<sub>1</sub>. Absorption spectra of cattle retinene<sub>1</sub> in chloroform and of the blue product which squid retinene<sub>1</sub> yields with antimony chloride, compared with similar preparations of synthetic retinene<sub>1</sub>. The absorption is plotted as extinction or optical density,  $\log I_o/I_o$  in which  $I_o$  is the incident and I the transmitted intensity (From Wald, 1947-48).

For this reason the proportions of vitamin  $A_1$  and manganese dioxide used in the procedure are important. If too much manganese dioxide is used, it adsorbs all the vitamin  $A_1$  at once, and oxidizes all of it to the 545 m $\mu$ -chromogen (WALD, 1947–48).

Once the nature of this reaction was appreciated, we recast it in more convenient form. The manganese dioxide powder is packed into a short column such as is used in chromatography. To oxidize 10 mg of vitamin  $A_1$ , about 0.6 g of manganese dioxide is employed. A solution of crystalline vitamin  $A_1$  in petroleum ether is poured in at the top of the column, and a solution of nearly pure retinene<sub>1</sub> is drawn off under light suction in the filtrate.

On washing through the column for a time with more petroleum ether, a high yield of retinene<sub>1</sub> is obtained. This can be freed of traces of contaminating substances by chromatographic adsorption on a column of calcium carbonate. It is adsorbed as a diffuse yellow zone, which travels slowly down the column on washing with petroleum ether, and is collected as an isolated fraction of high purity in the filtrate. The properties of this product are virtually identical with those of purified natural retinene<sub>1</sub> (Fig. 1).

References p. 228.

I have referred to this procedure as a *chromatographic oxidation*. The founder of chromatography, Michael Tswett, looked forward to the discovery of an entire class of such reactions, in which dry powders act at once as adsorbents and reagents, and I have no doubt that this is a correct view. Such reactions probably possess a degree of specificity and orientation not commonly realized in free solution, mimicking on occasion the character of enzymic processes. The range and properties of such chromatographic procedures deserve careful systematic examination.

#### THE COENZYME OF RETINENE REDUCTION\*

A simple procedure has been described for oxidizing vitamin  $A_1$  to retinene<sub>1</sub>. In the retina just the reverse process occurs: retinene<sub>1</sub> is reduced irreversibly to vitamin  $A_1$ .

Several years ago, as noted above, we brought this reaction into a cell-free preparation from cattle retinas. The retinas were frozen-dried, ground to a fine powder, and were extracted exhaustively with petroleum ether, all in darkness. The residue was stirred into a brei with neutral phosphate buffer. On exposing this to light, its rhodopsin was bleached, and the retinene  $_1$  so formed was converted almost completely to vitamin  $A_1$ .

In a study of the bleaching of rhodopsin in aqueous solution some years ago, we found that freshly prepared solutions undergo a special type of bleaching, which continues further than the bleaching of the same solutions after a period of aging (WALD, 1937–38). BLISS (1948) has lately reported that the basis of this extra bleaching in fresh rhodopsin solutions is the conversion of retinene<sub>1</sub> to vitamin  $A_1$ . We have confirmed this observation. A fresh rhodopsin solution, however, is not a satisfactory preparation in which to study the reduction of retinene<sub>1</sub>, for while this reaction is in progress, the enzyme system which accomplishes it is being rapidly inactivated, the vitamin  $A_1$  formed is being destroyed, and the intrusion of intermediates between rhodopsin and retinene<sub>1</sub> leaves equivocal the actual substrate in the process.

In order to analyse such systems further one would ordinarily attempt to fractionate them into their components. We had already begun such experiments when the investigation took a new turn with the discovery that the enzyme system can be fractionated anatomically through the structure of the retinal rods.

The vertebrate rod is composed of two sections, the inner and outer limbs or segments. The inner limb contains the nucleus, and is the principal seat of the ordinary cellular functions. The outer limb is a specialized outgrowth, which contains all the rhodopsin of the retina, and includes within its small compass the whole photoreceptor process.

When a retina is removed from the eye into Ringer solution with all possible care, the solution is found to contain large numbers of rod outer limbs which had broken off in the course of the dissection, just at their junctures with the inner segments. By scraping, one can break away about half the outer limbs from the surface of the retina, and collect them in a dense suspension, free from other retinal tissues, by filtration or differential centrifugation (Fig. 2).

When this procedure is carried out in dim red light, the outer limbs contain a large quantity of rhodopsin. On exposure to white light this bleaches; but in the isolated

A detailed account of the experiments reviewed in this section will be found in the paper of Wald and Hubbard (1948-49).

outer segment, unlike the whole retina, the retinene<sub>1</sub> which results is not converted to vitamin  $A_1$ . The isolated outer limb lacks some component of the system which performs this conversion.

It does not help this situation to suspend outer limbs in the presence of intact retinas. But if whole retinas are ground up in Ringer solution or phosphate buffer, though in the process almost all the outer segments are detached from other structures, the suspension which results does convert its retinene<sub>1</sub> efficiently to vitamin  $\Lambda_1$ . The crushing of the retinal cells releases substances which promote this process in the outer limbs.

If such a retinal brei is centrifuged at high speed and the clear, colourless supernatant solution is poured off, the solid residue — which retains all the rhodopsin — has lost the power to reduce retinene<sub>1</sub>. It regains this capacity on re-adding to it the supernatant. Furthermore, if one suspends isolated rod outer segments in such a water extract of crushed retinas, they now reduce their retinene<sub>1</sub> to vitamin  $A_1$ . The retinal extract supplies whatever the isolated outer limb lacks for performing this conversion (Fig. 3).

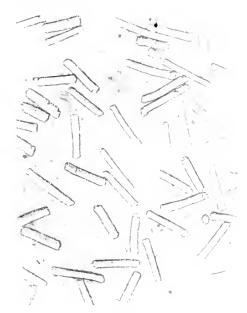
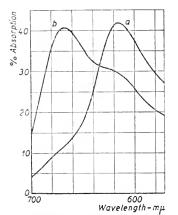


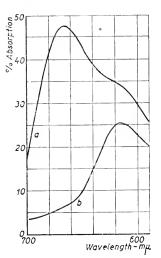
Fig. 2. Rod outer segments of the frog, suspended in Ringer solution. Magnification about 500 diameters. The longitudinal striations which can be seen in most of the outer limbs are characteristic of fresh preparations, and probably are evidence of a fibrillar structure. Later, cross-striations appear, and eventually dominate the structure; the first of these also are visible in the photograph (From Wald and Hubbard, 1948–49).

The water-soluble factor concerned with this process did not seem to involve a protein. It was relatively heat-stable, retaining most of its activity after boiling for as long as seven minutes. Also the ease and completeness with which it left the retinal tissue in a single extraction suggested that it was made up of small and relatively simple molecules — perhaps a coenzyme, or a hydrogen-donating substrate.



Now one would expect an enzyme protein to be relatively specific; and since retinene is found only in retinas,

Fig. 3. Rod outer limbs suspended in a water extract of retina convert retinene<sub>1</sub> to vitamin  $\Lambda_1$ ; washed retinal tissue is inactive. Isolated rod outer limbs were frozen-dried and preextracted with petroleum ether in the dark. Whole retinal tissue was ground, extracted with neutral phosphate buffer, and the outer limb material was suspended in the extract. Both this suspension and the washed retinal tissue were irradiated, incubated, and extracted with hexane. Spectra of the antimony chloride tests of these extracts are shown. That from the washed retinal tissue displays the band of unchanged retinene<sub>1</sub> (curve b); while the outer limb preparation suspended in retinal washings has converted its retinene<sub>1</sub> entirely to vitamin  $\Lambda_1$  (curve a). (From Wald and Hubbard, 1948–49).



its reductase might well be restricted to this tissue. A coenzyme or substrate, however, would ordinarily be unspecific, and one would expect to find it widely distributed among the tissues. This thought led us to try an extract of frog muscle as a suspension medium for rod outer limbs.

The preparation we used was the Muskelkochsaft — the

Fig. 4. Boiled muscle juice activates isolated rod outer limbs. Equal numbers of rod outer segments were suspended in phosphate buffer and in a boiled juice of frog muscle. The suspensions were exposed to light, left at room temperature for I hour, and the residues extracted with petroleum ether. The spectra of the antimony chloride tests with these extracts are shown. The outer limbs in buffer had failed to convert their retinene, to vitamin A, (curve a); those suspended in boiled muscle juice had done so completely (curve b). The relatively low content of vitamin  $A_1$  shown in curve b is due to its destruction in

preparations of this type. (From Wald and Hubbard, 1948-49).

boiled muscle juice — of MEYERHOF (1918). Rod outer segments suspended in this medium converted their retinene, quantitatively to vitamin A<sub>1</sub> (Fig. 4).

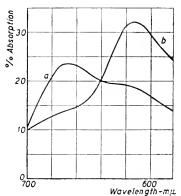
Boiled muscle juice contains a number of substances which could donate hydrogen for the reduction of retinene<sub>1</sub>. It also contains a major coenzyme of hydrogen transfer, cozymase, Coenzyme I, or DPN.

When rod outer limbs were suspended in a buffer solution to which DPN had been added, they failed to transform their retinene, to vitamin A<sub>1</sub>. But if they—or an inactive preparation of washed retinal tissue—were provided with reduced cozymase, DPN-H<sub>2</sub>, they performed this conversion quantitatively (Fig. 5).

Given a proper substrate, rod outer limbs can themselves reduce cozymase. We have found a first such substrate in fructose diphosphate. Rod outer segments suspended in a solution to which both DPN and fructose diphosphate were added converted their retinene<sub>1</sub> completely to vitamin  $A_1$ . The outer segments must therefore contain an enzyme system for reducing DPN when a suitable hydrogen donor is made available. It is highly improbable that fructose diphosphate itself is the source of hydrogen in this reaction. More probably the outer limbs also possess the enzyme aldolase, which cleaves fructose diphosphate to yield 3-glyceraldehyde phosphate, the normal substrate for the reduction of DPN in the lactic acid fermentation.

The conversion of retinene, to vitamin A, is therefore a coupled reduction in which DPN-H<sub>2</sub> acts as coenzyme. The essential process is the transfer of two

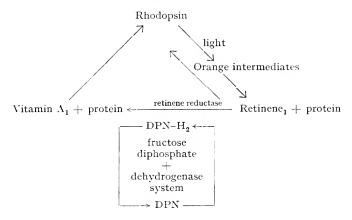
Fig. 5. The action of reduced cozymase on washed retina. Equal portions of a preparation of water-extracted frog retina were suspended in a solution containing reduced DPN, and in an otherwise identical solution lacking only the DPN-H<sub>2</sub>. Both suspensions were bleached in the light, incubated, and the residues extracted with petroleum ether. Spectra of the antimony chloride tests with these extracts are shown. The control preparation yielded retinene, alone (curve a); while in the washed retina to which reduced DPN had been added this had been converted almost completely to vitamin  $A_1$  (curve b). (From Wald and Hubbard, 1948-49).



hydrogen atoms from  $DPN-H_2$  to retinene<sub>1</sub>, reducing its aldehyde group to the primary alcohol group of vitamin  $A_1$ . We may assume that in this process an apoenzyme, retinene reductase, still to be revealed, takes part. The reaction may be written:

$$C_{19}H_{27}CHO + DPN-H_2 \xrightarrow{\text{retinene reductase}} C_{19}H_{27}CH_2OH + DPN \text{ retinene}_1 \text{ vitamin } A_1$$

In the rod outer limb this system works in conjunction with a second dehydrogenase system which reduces DPN, using a derivative of fructose diphosphate as hydrogen donor. The total process may be formulated:



THE RETINENE REDUCTASE SYSTEM\*

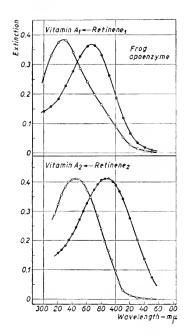
With the coenzyme, the first component of the retinene reductase system was defined. Up to this point the apoenzyme had remained a matter of surmise, buried in the structure of the rod outer limb. The substrate had been obtained by bleaching rhodopsin, and was both equivocal in character and very limited in quantity.

The nature of the substrate was resolved with the observation that for this one could use pure synthetic retinene<sub>1</sub> prepared as described above by the chromatographic oxidation of crystalline vitamin  $A_1$  on manganese dioxide. Retinene<sub>1</sub> is fat-soluble, and was originally introduced into the system with the aid of digitonin, with which it forms a water-soluble complex. Later the digitonin proved to be unnecessary, for reasons to be discussed below.

The apoenzyme was found to be readily extracted with dilute salt solutions from homogenates of frog or cattle retinas, forming clear, almost colourless solutions. Though the apoenzyme has not yet been isolated as a pure substance, it has been separated from the other components of the system and some of its properties have been determined. It is precipitated by half-saturated ammonium sulphate and re-dissolves without losing its activity. It is retained by a Visking membrane, and survives dialysis for 18 hours at 5° C against neutral phosphate buffer. It is destroyed by heating at 100° within 30 seconds. Its  $p_{\rm H}$  optimum lies at about 6.5.

The retinene reductase system can therefore now be assembled from its separate components, all in true solution: the coenzyme, DPN-H<sub>2</sub>, prepared by the method

<sup>\*</sup> A short account has been published of the experiments which follow (WALD, 1949). A more complete description of these experiments will appear in the *Journal of General Physiology*.

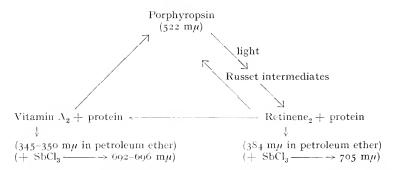


of Ohlmeyer (1938); the substrate, synthetic retinene<sub>1</sub>; and the apoenzyme, contained in a clear, almost colourless extract of homogenized frog or cattle retinas. When these three components are mixed and incubated for  $\mathbf{1}$ - $\mathbf{2}$  hours at room temperature, the retinene<sub>1</sub> is quantitatively reduced to vitamin  $\Lambda_1$  (Fig. 6, upper half).

Fig. 6. The action of frog retinene reductase on synthetic retinene<sub>1</sub> and retinene<sub>2</sub>. Each of the experimental mixtures included a synthetic retinene dissolved in 1% digitonin, 0.7 mg of reduced cozymase per ml, 5.5 mg of nicotinamide per ml, and extracts of homogenized frog retinas in m/30 phosphate buffer, p<sub>H</sub> 6.81. The controls differed only in that the retinal extracts were replaced with either the same extract which had been boiled for ½ minute (upper figure) or with the phosphate buffer alone (lower figure). The enzyme and control mixtures were incubated together for 2 hours at 23° C. Methanol was added to each to a concentration of 60%, and they were extracted with hexane. The spectra of the hexane extracts are shown. Those from the controls (solid circles) show the spectra of the unaltered retinenes; those from the enzyme mixtures (open circles) show complete conversion to the corresponding vitamins A.

#### RETINENE, AND VITAMIN As; SPECIFICITY OF RETINENE REDUCTASE

In the rods of freshwater fishes, cyclostomes and certain amphibia, rhodopsin is replaced by the purple, light-sensitive porphyropsin. This takes part in a retinal cycle identical in form with the rhodopsin system, but based upon the new carotenoids, retinene, and vitamin A<sub>2</sub> (WALD, 1937; 1945–46):



The structure of vitamin  $A_2$  is still uncertain. It seems clear, however, that like  $A_1$  it is a primary alcohol; and that retinene<sub>2</sub>, as emerges from experiments of Morton *et al.* and from those discussed below, is in all probability its aldehyde.

Morton, Salah, and Stubbs (1946) reported that when solutions of vitamin A<sub>2</sub> in petroleum ether are let stand in the cold over solid manganese dioxide, the vitamin is replaced by a product resembling retinene, in spectrum and antimony chloride reaction. They found that this product forms, as does retinene<sub>1</sub>, a 2-4-dinitrophenylhydrazone, indicating the presence of a carbonyl group. That this substance possesses a conjugated carbonyl group is shown also by a large displacement of its spectrum in

ethanol as compared with hexane (cf. Wald, 1947–48). That the carbonyl group replaces the primary alcohol group of vitamin  $A_2$  is shown by the fact that though the vitamin is hypophasic, its oxidation product is epiphasic in partition between hexane and 90% methanol. This information, together with what follows, leaves little doubt that this product is retinene, and that it is the aldehyde of vitamin  $A_2$ .

As in the manufacture of retinene<sub>1</sub>, we have found that the oxidation of vitamin  $\Lambda_2$  to retinene<sub>2</sub> can be carried out conveniently in chromatographic form. The procedure is identical with that used in making retinene<sub>1</sub>; but in this case only about half as much manganese dioxide is employed — 0.3 g to oxidize 10 mg of vitamin  $\Lambda_2$ . The yield of retinene<sub>2</sub> is in the neighbourhood of 50%; and it can be brought to a state of high purity by chromatographic adsorption on a column of calcium carbonate.

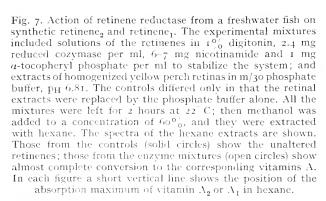
In our past experience one of the most remarkable properties of the porphyropsin system has been its detailed parallelism in chemical behaviour with the rhodopsin cycle. In the present instance this parallelism is maintained, for retinene<sub>2</sub> is reduced to vitamin  $A_2$  by an enzyme system entirely similar to that which reduces retinene<sub>1</sub>.

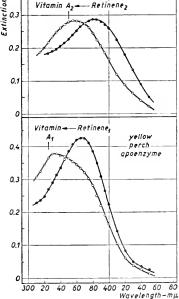
This system can be assembled from the following components: the coenzyme, DPN-H<sub>2</sub>; the substrate, synthetic retinene<sub>2</sub>, prepared by the chromatographic oxidetion of vitamin A<sub>2</sub> on manganese dioxide; and the apoenzyme, contained in a clear, almost colourless saline extract of homogenized freshwater fish retinas (yellow perch, sunfish). When these three components are mixed and left at room temperature for two hours, the retinene<sub>2</sub> is reduced almost entirely to vitamin A<sub>2</sub> (Fig. 7, upper half).

Since the coenzyme of retinene reduction is common to the rhodopsin and porphyropsin cycles, one may inquire into the specificity of the apoenzyme. To test this, experiments were performed in which the frog apoenzyme was allowed to act on retinene, and the fish apoenzyme on retinene. It emerged that the reduction proceeded as smoothly and completely with the crossed as with the normal substrates (Figs 6 and 7).

There is no reason therefore to designate the apoenzyme differently in the rhodopsin and porphyropsin systems. We have to deal with a single apoenzyme, retinene reductase, which with the single coenzyme, dihydrocozymase, reduces either of the retinenes to the corresponding vitamin A.

This enzyme system introduces a new vitamin into the chemistry of rod vision, for the central component





of cozymase is nicotinamide, the anti-pellagra factor of the vitamin B complex. It presents also the novel phenomenon of widely distinct vitamins not only interacting *in vitro*, but of one of them participating directly in the regeneration of the others. I do not know a comparable relation in the whole of biochemistry.

#### STABILITY

It has been known for some time that animal and certain plant tissues contain a nucleotidase which cleaves cozymase and dihydrocozymase, and which is released into homogenates and tissue breis by the breaking of the cells. Measurements made on various tissues of the rat have shown this enzyme to be particularly active in brain, to which of course retina is closely related (Mann and Quastel, 1941; Handler and Klein, 1942). The action of this enzyme makes a number of the preparations which we have described unstable.

It was noted above that solutions of rhodopsin, prepared by extracting fresh retinal tissue with water solutions of digitonin, rapidly lose the power to reduce retinene<sub>1</sub>. Within 3-4 hours their ability to perform this process usually falls to very low levels. The principal cause of this failure is the loss of cozymase.

This is shown by the following experiment. A freshly prepared rhodopsin solution was kept at about 23° C for 18 hours. At the end of this period it was divided into halves, and to one half reduced cozymase was added (1.5 mg per ml). Both portions were bleached in the light and were incubated for 1 hour. The control portion converted no more than a trace of its retinene<sub>1</sub> to vitamin A<sub>1</sub>; that to which DPN-H<sub>2</sub> was added had completed this conversion. It is clear that the apoenzyme in such preparations is relatively stable; their loss of activity is caused by the destruction of the coenzyme.

Cozymase and reduced cozymase are protected from the action of the nucleotidase by the presence of free nicotinamide (2–20 mg per ml) (Mann and Quastel, 1941; Handler and Klein, 1942). It has recently been reported also that a-tocopheryl phosphate (about 1 mg per ml) similarly protects cozymase (Spaulding and Graham, 1947).

The nucleotidase has been reported to be in general insoluble in water or dilute salt solutions. Our experiments show that it does go into solution in the 2% digitonin with which we extract rhodopsin. It also is active in all our retinal homogenates and particulate preparations. Whether it enters the saline extracts which contain our apoenzyme we have not yet determined. A number of our fish enzyme preparations have definitely been unstable, but they also tend to be slightly turbid, and may contain small amounts of very fine particles.

In any case we have taken the precaution ordinarily to add nicotinamide to our enzyme preparations; and to those from freshwater fish retinas, in which the nucleotidase appears to be particularly active, we have added also  $\alpha$ -tocopheryl phosphate.

These adjustments extend still further the participation of vitamins in the retinene reductase system. Nicotinamide acts not only as the key component of the cozymase molecule, but in the free condition protects cozymase from destruction. In this action it is aided by vitamin E phosphate. As many as three vitamins therefore interact with one another in this single system.

#### THE STATE OF THE RETINENES

With the first use of the synthetic retinenes as substrates there arose the problem how, as typically fat-soluble substances, they were to be introduced into the aqueous *References p. 228*.

enzyme system. This was solved initially by bringing the retinenes into water solution with the aid of digitonin, with which they form water-scluble complexes.

The use of digitonin, however, proved to be unnecessary. The retinal extracts which contain the apoenzyme take up the retinenes directly. If either of the retinenes is concentrated in a few drops of petroleum ether, and is agitated together with a water extract of retinas while the last of the petroleum ether is drawn off under suction, the retinenes gradually are taken up to yield clear yellow solutions. This is one indication that the retinenes couple with water-soluble substances from the retina. Primarily in these preparations they attach to protein, for they are precipitated from such solutions with the protein fraction.

It has been known for some time that in the product of bleaching rhodopsin in solution, most of the retinene<sub>1</sub> is found loosely coupled with protein (Wald, 1937–38, pp. 812–813). In this condition it behaves as a p<sub>H</sub> indicator, deep yellow in acid and almost colourless in alkaline solution; hence Lythgoe's proposal that it be called "indicator yellow". Synthetic retinene<sub>1</sub> does not change its spectrum at all with p<sub>H</sub>; nor does natural retinene<sub>1</sub> after partial purification by adsorption and elution (Wald, 1947–48). Ball et al. have now shown that the p<sub>H</sub> indicator property is characteristic of retinene<sub>1</sub> in the coupled condition (Ball, Collins, Morton, and Stubbs, 1948). Retinene<sub>1</sub> condenses spontaneously, as do aldehydes generally, with a variety of amino compounds — proteins, amino acids, aromatic amines — and in this state acts as a p<sub>H</sub> indicator. Indeed a second evidence that the synthetic retinenes added directly or in digitonin solution to our apoenzyme extracts couple with other molecules is that they have acquired this property. They have in fact come to resemble closely the natural products of bleaching rhodopsin and porphyropsin in solution.

A third evidence that synthetic retinene<sub>1</sub> couples with other molecules in our enzyme system is that it becomes more and more difficult to extract with fat solvents as the mixture is made more alkaline. If to a solution of retinene<sub>1</sub> in digitonin one adds methanol in a final concentration of 60% and shakes vigorously with petroleum ether, almost all the retinene enters the petroleum ether regardless of the p<sub>H</sub>. But if retinene<sub>1</sub> in digitonin is mixed with a water extract of the retina prior to carrying out this procedure, smaller and smaller fractions of the retinene enter the petroleum ether as the alkalinity is increased. At p<sub>H</sub> 4 about 2/3 of the retinene is extracted with petroleum ether in one partition; at p<sub>H</sub> 9 only about 1/6 of the retinene is extracted. What this probably means is that since retinene<sub>1</sub> is coupled by the condensation of its carbonyl group with the amino groups of other molecules, alkalinity favours this process by increasing the proportion of free amino groups, while acidity hinders it by converting amino groups to ammonium ions\*.

The net result of these considerations is that we must regard the normal state of the retinenes in retinas and retinal extracts as a labile equilibrium between free molecules and those loosely coupled to other substances. There is no unique retinal molecule. however, with which the retinenes couple and which therefore should be designated "visual yellow" or "indicator yellow". On the contrary, the retinenes regularly condense with a variety of molecules, some protein, some forming fat-soluble complexes. So, for example, when the retinenes have been extracted from retinas with petroleum ether,

<sup>\*</sup> On observing that retinene<sub>1</sub> is not readily extracted with petroleum ether from alkaline solutions of bleached rhodopsin, BLISS (1948) concluded that it had not been formed. It is formed, but like added retinene<sub>1</sub> it is retained by coupling with other retinal molecules.

and are hence protein-free, they still behave as  $p_H$  indicators, and are therefore still in the coupled condition.

Not only do the retinenes form a variety of retinal complexes, but normally they migrate from one such association to another. One such migration is established by the present experiments. Rhodopsin and retinene reductase are different proteins. Retinene originates on rhodopsin protein, but it must transfer to the reductase protein preparatory to its reduction. Retinene, is involved in a like situation. Such changes of the molecules with which the retinenes are coupled must play an important part in retinal metabolism.

#### SUMMARY

The retinene<sub>1</sub> which results from the bleaching of rhodopsin now appears to be vitamin  $A_1$  aldehyde. Morton *et al.* have given the best evidence for this, and have shown that retinene<sub>1</sub> can be prepared by the mild oxidation of vitamin  $A_1$ . A simple procedure is described for performing this process chromatographically on a column of manganese dioxide.

In the retina, retinene<sub>1</sub> is converted irreversibly to vitamin  $A_1$  by an enzyme system in which reduced cozymase (reduced Coenzyme I, DPN-H<sub>2</sub>) serves as coenzyme. The essential process is the transfer of two hydrogen atoms from DPN-H<sub>2</sub> to retinene<sub>1</sub>, reducing its aldehyde group to the primary alcohol group of vitamin  $A_1$ .

The enzyme system which performs this reduction can be assembled in solution from the following components: the coenzyme,  $\mathrm{DPN-H_2}$ ; as substrate, synthetic retinene<sub>1</sub>; and the apoenzyme extracted with dilute salt solutions from homogenized frog or cattle retinas. The apoenzyme is non-dialysable, is precipitated by half-saturated ammonium sulphate, and is destroyed by heating at 100° C within 30 seconds. Its  $p_{\mathrm{H}}$  optimum lies at about 6.5.

In the rods of freshwater fishes, a parallel enzyme system reduces retinene<sub>2</sub> to vitamin  $\Lambda_2$ . This can be assembled from the following components, all in true solution: the coenzyme, DPN-H<sub>2</sub>; as substrate, synthetic retinene<sub>2</sub>, prepared by the chromatographic oxidation of vitamin  $\Lambda_2$  on manganese dioxide; and the apoenzyme extracted with dilute salt solutions from freshwater fish retinas (sunfish, yellow perch).

The apoenzyme from frog retinas reduces retinene<sub>2</sub> as effectively as retinene<sub>1</sub>. Similarly the fish apoenzyme acts equally well upon both retinenes. One need consider only one apoenzyme, retinene reductase, which together with one coenzyme, DPN-H<sub>2</sub>, reduces either of the retinenes to the corresponding vitamin A.

The retinene reductase system brings a second vitamin into the chemistry of rod vision. It presents the novel phenomenon of one vitamin regenerating another, for the central component of DPN-H<sub>2</sub> is nicotinamide, the anti-pellagra factor of the vitamin B complex.

Rhodopsin solutions and retinal homogenates rapidly lose their power to reduce the retinenes, through destruction of their DPN by a nucleotidase. Rhodopsin solutions which have lost their activity in this way are re-activated by the addition of new DPN-H<sub>2</sub>. The coenzyme can also be protected by the presence of free nicotinamide and of a-tocopheryl phosphate.

On addition to the enzyme system, the synthetic retinenes rapidly couple with other molecules, and primarily with protein. The normal state of the retinenes in retinas and retinal extracts is a labile equilibrium between the free and the coupled condition. The retinenes couple with a variety of retinal molecules, and migrate freely from one to the other.

#### RÉSUMÉ

Le rétinène<sub>1</sub>, qui résulte du blanchissement de la rhodopsine, apparaît maintenant comme étant l'aldéhyde de la vitamine A<sub>1</sub>. Morton et collab, en ont donné la meilleure preuve en montrant que le rétinène<sub>1</sub> peut être préparé par une oxydation ménagée de la vitamine A<sub>1</sub>. Un procédé simple est décrit, qui permet d'effectuer cette opération par chromatographie sur une colonne de bioxyde de manganèse

Dans la rétine, le rétinène<sub>1</sub> est converti irréversiblement en vitamine  $A_1$  par un système enzymatique dans lequel la cozymase I réduite (DPN-H<sub>2</sub>) sert de coenzyme. Le processus consiste essentiellement en un transfert de deux atomes d'hydrogène du DPN-H<sub>2</sub> sur le rétinène<sub>1</sub>, réduisant sa fonction aldéhydique en fonction alcoolique primaire de la vitamine  $A_1$ .

Le système enzymatique qui effectue cette réduction peut être constitué en solution à partir

des composantes suivantes: la coenzyme, DPN- $H_2$ ; comme substratum du rétinène<sub>1</sub> synthétique; et l'apoenzyme, extraite de rétines homogénéisées de grenouilles ou de bœufs au moyen de solutions salines diluées. L'apoenzyme n'est pas dialysable; elle est précipitée par le sulfate d'ammonium à demisaturation et détruite par chauffage à 100° pendant 30 secondes. Son  $p_H$  optimum est d'environ 6.5.

Dans les bâtonnets de la rétine de poissons d'eau douce, il existe un système enzymatique parallèle, qui réduit le rétinène, en vitamine  $A_2$ . Ce système peut être constitué à partir des composantes suivantes, toutes en vraie solution: la coenzyme, DPN-H<sub>2</sub>; comme substratum, du rétinène, synthétique, préparé par oxydation chromatographique de la vitamine  $A_2$  au bioxyde de manganèse; et l'apoenzyme, extraite au moyen de solutions salines diluées à partir de rétines homogénéisées de poissons d'eau douce (poisson-soleil, perche jaune).

L'apoenzyme de la rétine de grenouille réduit le rétinène, aussi bien que le rétinène<sub>1</sub>. De même, l'apoenzyme de poissons d'eau douce agit également bien sur les deux rétinènes. Il n'est donc besoin de considérer qu'une seule apoenzyme, la réductase du rétinène, qui, en présence d'une coenzyme,

le DPN-H2, réduit l'un ou l'autre des deux rétinènes en la vitamine A correspondante.

Le système de la réductase du rétinène introduit une seconde vitamine dans la chimie de la vision par bâtonnets. Il présente le phénomène nouveau d'une vitamine qui en régénère une autre, attendu que la composante essentielle du DPN-H<sub>2</sub> est la nicotamide, le facteur antipellagreux du complexe vitamique B.

Des solutions de rhodopsine et d'extraits homogénéisés de rétines perdent rapidement leur pouvoir de réduire les rétinènes, de par la destruction de leur DPN par une nucléotidase. Des solutions de rhodopsine ayant ainsi perdu leur pouvoir réducteur sont réactivées par l'addition d'une quantité fraîche de DPN-H<sub>2</sub>. La coenzyme peut également être protégée par la présence de nicotamide libre et de phosphate d'a-tocophéryle.

En plus du système enzymatique étudié, les rétinènes synthétiques forment des produits d'addition avec d'autres molécules, et spécialement avec les protéines. L'état normal des rétinènes dans les rétines et leurs extraits est un équilibre labile entre la forme libre et la forme associée. Les rétinènes s'associent avec une variété de molécules rétinales et migrent librement de l'une à l'autre.

#### ZUSAMMENFASSUNG

Das Retinen, welches bei der Bleichung des Rhodopsins entsteht, entpuppt sich jetzt als Vitamin  $A_1$ -Aldehyd. Morron und Mitarb, haben dafür den besten Beweis geliefert, dadurch dass sie gezeigt haben dass Retinen, durch milde Oxydation von Vitamin  $A_1$  gebildet werden kann. Es wird eine einfache Prozedur beschrieben, um diesen Vorgang chromatographisch mittels einer Mangandioxyd-Säule zu bewerkstelligen.

In der Netzhaut wird Retinen, irreversibel in Vitamin A, verwandelt durch ein Enzymsystem in welchem reduzierte Cozymase I (DPN-H<sub>2</sub>) als Coenzym dient. Die Hauptreaktion besteht dabei in der Übertragung von zwei Wasserstoffatomen vom DPN-H<sub>2</sub> auf das Retinen, dessen Aldehyd-

gruppe zur primären Alkoholgruppe des Vitamins A<sub>1</sub> reduziert wird.

Das Enzymsystem welches diese Reduktion vollführt, kann in Lösung aus folgenden Komponenten zusammengestellt werden: das Coenzym, DPN-H<sub>2</sub>; als Substrat, synthetisches Retinen<sub>1</sub>; und das Apoenzym, welches durch verdünnte Salzlösungen aus homogenisierten Frosch- oder Rinder-Netzhäuten ausgezogen wird. Das Apoenzym ist nicht dialysierbar; es wird durch halbgesättigte Ammoniumsulfat-Lösung gefällt und durch Erhitzen auf 100° innerhalb 30 Sek. zerstört. Sein p<sub>H</sub> Optimum liegt bei ca 6.5.

In den Stäbchen von Süsswasserfischen besteht ein paralleles Enzymsystem, welches Retinen $_2$  zu Vitamin  $A_2$  reduziert. Es kann aus folgenden, alle in wahrer Lösung befindlichen Komponenten zusammengestellt werden: das Coenzym, DPN- $H_2$ ; als Substrat, synthetisches Retinen $_2$ , durch chromatographische Oxydation von Vitamin  $A_2$  an Mangandioxyd dargestellt; und das Apoenzym, welches durch verdünnte Salzlösungen aus den Netzhäuten von Süsswasserfischen (Sonnenfisch, gelber Barsch) ausgezogen wird.

Das Apoenzym aus Froschnetzhäuten reduziert Retinen<sub>2</sub> so wirksam wie Retinen<sub>1</sub>. Desgleichen wirkt das Fisch-Apoenzym gleich gut an beiden Retinenen. Man hat also nur ein einziges Apoenzym zu betrachten, die Retinen-Reduktase, welche zusammen mit einem Coenzym, dem DPN-H<sub>2</sub>, beide

Retinene zu den entsprechenden A-Vitaminen reduziert.

Das System der Retinen-Reduktase führt ein zweites Vitamin in die Chemie des Stäbchen-Sehens ein. Es zeigt das neuartige Phänomen eines Vitamins welches ein anderes regeneriert, denn die wichtigste Komponente vom DPN-H<sub>2</sub> ist das Nikotinamid, der Antipellagra-Faktor des Vitamin B-Komplexes.

Lösungen von Rhodopsin und homogenisierten Netzhautextrakten verlieren rasch ihr Vermögen, Retinene zu reduzieren; ihr DPN wird nämlich von einer Nukleotidase zerstört. Auf solche Art inaktivierte Rhodopsin-Lösungen können durch Zugabe von DPN- $H_2$  reaktiviert werden. Das

Coenzym kann auch durch die Gegenwart von freiem Nikotinamid oder von  $\alpha$ -Tokopherylphosphat geschützt werden.

Ausser mit dem Enzymsystem, verbinden sich die synthetischen Retinene auch rasch mit anderen Molekülarten, besonders mit Proteinen. Der Normalzustand der Retinene in der Netzhaut und in Netzhautextrakten ist ein labiles Gleichgewicht zwischen freier und gebundener Substanz. Die Retinene wandern leicht von einer zur anderen der verschiedenen in der Netzhaut befindlichen Molekeln mit denen sie lose Verbindungen eingehen.

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# EXPERIMENTELLE BINDUNG VON EIWEISSKÖRPERN AN ZELLKERNE UND NUKLEINSÄUREN

(KURZE MITTEILUNG)

von

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Ι

Zu einem Reaktionsansatz der Prostataphosphatase mit Glycerinphosphat bei  $p_H=3.7$  haben wir isolierte Zellkerne der Thymusdrüse zugesetzt und eine Hemmung des Ferments auf etwa die halbe Wirkung beobachtet. Bei  $p_H=5$  und ebenso in Gegenwart gewisser (verdrängender) Eiweisskörper bleibt die Hemmung aus (Tab. I).

TABELLE I

ZELLKERNE HEMMEN DAS FERMENT
(PROTEIN E IST EINE FRAKTION AUS MUSKULATUR)

|                                 | рн = 3.7 |    |     |     |                    | pH = 5.0 |      |
|---------------------------------|----------|----|-----|-----|--------------------|----------|------|
| Kerne (γ)                       |          | 35 | 140 | 280 | (+ 15 ? Protein E) |          | 3500 |
| Fermentwirkung<br>(rel. Zahlen) | 100      | 85 | 61  | 49  | 102                | 130      | 135  |

Die Hemmung beruht auf einer Bindung des Ferments an den Zellkern; die schützende Wirkung des Proteins auf einer Verdrängung. Die Verbindung lässt sich durch Zentrifugieren abtrennen; unter passenden Bedingungen wird dabei ein fermentfreier Überstand erhalten. Der Niederschlag hat dann eine der Hemmung entsprechende Fermentwirkung, die durch Zugabe von Eiweiss auf etwa die ursprüngliche Höhe gebracht werden kann.

П

Die Wirkung der Zellkerne beruht auf ihrem Gehalt an Nukleinsäure. Auch Thymonukleinsäure bildet eine Verbindung mit dem Ferment, die p<sub>H</sub>-abhängig ist und durch andere Eiweisskörper gelöst werden kann. In dieser Bindung hat das Ferment, unter der (nicht völlig exakten) Annahme einer linearen Zeitfunktion, eine minimale Restwirkung von 13%.

Ein ähnlicher Rest wurde bei  $\alpha$ -Glycerinphosphat, bei  $\beta$ -Glycerinphosphat und bei

Adenylsäure als Substrat gefunden. Er zeigt sich ebenfalls bei den Phosphatasen aus Muskel und aus weiblichem Harn. Er bleibt ferner erhalten, wenn das Prostataferment durch grössere Mengen Hefenukleinsäure oder durch Tannin gehemmt wird. Pikrinsäure und Nikotin haben unter analogen Bedingungen keine Wirkung. Der Rest ist unabhängig von der Substratkonzentration und vom p<sub>H</sub> in den Grenzen 2.5 und 4.0.

Werden  $3.7 \gamma$  Ferment bei  $p_H = 3.7$  ohne Zusatz und mit  $2 \gamma$  Nukleinsäure 20 min auf der Ultrazentrifuge bei 115000 g zentrifugiert, so bleibt ein geringer Anteil des Ferments in Lösung, der hemmbar ist wie das gesamte Ferment (Tab. II). Hieraus und aus der Restwirkung der Verbindung des Ferments mit Zellkernen geht hervor, dass nicht 13% des Ferments ungebunden bleiben, sondern dass das gebundene Ferment einen Wirkungsrest von 13% behält.

TABELLE II
DIE VERBINDUNG FERMENT-NUKLEINSÄURE IN DER ULTRAZENTRIFUGE

|        |                          | Über | stand |     |  |  |
|--------|--------------------------|------|-------|-----|--|--|
| Nicht  | Nukleinsäure (γ)         |      |       |     |  |  |
| entri- | #0.00.000                | 2    | 2     | 60  |  |  |
| ugiert | p <sub>H</sub> im Ansatz |      |       |     |  |  |
|        | 4.8                      | 4.8  | 3.7   | 4.8 |  |  |
| 100    | 102                      | 11   | 2     | 0   |  |  |

Zu Ansätzen von 8  $\gamma$  Ferment mit der maximal hemmenden Nukleinsäuremenge (o.8  $\gamma$ ) wurde eine Anzahl von Eiweisstoffen in Verdünnungsreihen zugegeben und so die Menge ermittelt, welche die Hemmung auf das halbe Maximum erniedrigt. In Tab. III ist diese Menge in Mikrogramm angegeben.

TABELLE III ENTHEMMUNG DURCH EIWEISSKÖRPER

| Substanz              | γ         | Substanz                   | 7'  |  |
|-----------------------|-----------|----------------------------|-----|--|
| Gliadin               | 700       | Scrumglobulin              | 5   |  |
| Pepsin                | ; (> 400) | Hämoglobin                 | 2.8 |  |
| Tabakınosaikvirus     | 36        | Salmin                     | 2.4 |  |
| Eicralbumin           | 25        | Protamin aus Heringssperma | 0.7 |  |
| Inaktiviertes Ferment | 2.4       | Protein E                  | 0.3 |  |

Das Tabakmosaikvirus wurde geprüft, weil die Frage war, ob sein Nukleotidanteil hemmen oder sein Proteinanteil enthemmen würde. Dass (sauer) inaktiviertes Ferment enthemmen würde, war zu postulieren; seine Wirkung wird durch Pepsinverdauung zerstört. Die Verbindung Nukleinsäure-Serumglobulin wurde in grösseren Ansätzen gravimetrisch bestimmt und zeigte das konstante Gewichtsverhältnis 1:3.

#### ZUSAMMENFASSUNG

Phosphatase wird durch Bindung an Zellkerne oder an Nukleinsäure stark gehemmt. Die Verbindung ist nur bei  $p_H < 5$  beständig. Durch Eiweisstoffe kann das Ferment verdrängt und wieder mit der ursprünglichen Aktivität erhalten werden.

#### SUMMARY

Phosphatase is strongly inhibited by combination with cell nuclei or with nucleic acids. The compound is only stable at  $p_H$ -values less than 5. The enzyme can be displaced by proteins and recovered with the original activity.

#### RÉSUMÉ

La phosphatase est fortement inhibée par combinaison avec les noyaux cellulaires au avec les acides nucléiques. Cette combinaison n'est stable qu'à un p<sub>H</sub> inférieur à 5. Au moyen de protéines le ferment peut être déplacé de cette combinaison et régénéré avec son activité primitive.

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# THE BIOLOGICAL INCORPORATION OF PURINES AND PYRIMIDINES INTO NUCLEOSIDES AND NUCLEIC ACID

by

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#### INTRODUCTION

The fundamental studies by MEYERHOF and his associates on the metabolism of phosphoric esters in muscle extracts marked the beginning of a very fruitful era in which the pathway of breakdown and synthesis of carbohydrates gradually became known. Meyerhof showed that Harden and Young's discovery of phosphate uptake in cell-free yeast fermentation mixtures could be extended to animal tissues, especially muscle. Later Meyerhof and his associates and Needham and Pillai in Cambridge showed that esterification of phosphate in muscle was coupled to the oxidation-reduction between phosphotriose and cozymase. This development led to the discovery of the acylphosphates (Warburg and coworkers, Lipmann). It was known, however, from LUNDSGAARD's studies that muscle, performing alactacid contractions in an oxygen-free atmosphere accumulates large amounts of hexosephosphoric esters. This is further accentuated if dinitrophenol which 'uncouples' oxidative-reductive phosphorylation is added together with iodoacetate. These observations which were made by CORI AND Cort in 1936 indicated that phosphate can also be incorporated into ester linkage by another process which has nothing to do with oxidation-reduction. The phenomenon of phosphate uptake independent of oxidation-reduction was very soon encountered in in vitro experiments too. Within the same year PARNAS AND OSTERN reported that the glycogen present in aged and dialysed muscle extracts can react with inorganic phosphate. A few months later Carl and Gerty Cori isolated α-glucose-l-phosphate from muscle extracts and three years later CORI, CORI, AND SCHMIDT demonstrated the synthesis of a polysaccharide from  $\alpha$ -glucose-l-phosphate by means of a muscle enzyme. Kiessling, a student of MEYERHOF, performed independently in 1939 an analogous in vitro synthesis of polysaccharide using a yeast enzyme. During the subsequent years Cori and his associates turned their attention towards the kinetics of starch and glycogen synthesis in vitro. A number of important studies on starch, dextran and sucrose formation in enzyme systems from plants and microorganisms appeared during the next three or four years. The studies on the enzymatic synthesis of ribo- and desoxyribonucleosides can also be considered an outgrowth of Cort's fundamental observations on phosphorolysis of glucosidic linkages.

References p. 237.

#### ENZYMATIC SYNTHESIS OF PURINE RIBO-NUCLEOSIDES

The presence in animal tissues of an enzyme, called nucleosidase which splits of purines from purine nucleosides of the ribose series has been known for many years. KLEIN<sup>1</sup> who made a detailed study of this enzyme found that phosphate and arsenate enhance the enzymatic splitting of purine nucleosides. When I spent some time in 1943-1944 isolating nucleosidases from liver it was done only with the purpose of using these enzymes as analytical tools in an optical micromethod which I was trying to develop at that time. I had no knowledge about Klein's work at the time when I came across the observation that nucleosidase subjected to prolonged dialysis loses its activity. In view of observations by Meyerhof and Cori it was not too far-fetched to try to add inorganic ortho-phosphate to the system and it turned out that this addition completely restored the catalytic activity of the system. Pursuing the analogy to Cori's work on the polysaccharide phosphorylase<sup>2</sup> I attempted to demonstrate the formation of ribosel-phosphate as a suspected intermediate. These attempts failed quite a few times. Fortunately Lowry who was my colleague at that time at The Public Health Research INSTITUTE had worked out a new method for phosphate determination which operates at p<sub>H</sub> 4. This method, the well-known Lowry-Lopez method<sup>3</sup>, permits an estimation of highly labile phosphoric esters such as phosphocreatine and acylphosphates in the presence of inorganic phosphate. With the LOWRY-LOPEZ procedure it became possible to show a clearcut proportionality between liberation of purine and uptake of inorganic phosphate<sup>4</sup>. It was fairly obvious therefore that a new and highly acid-labile phosphoric ester was formed as a product of the enzymatic phosphorolysis of nucleosides. The ester was later obtained as the barium salt. It contained I mole pentose for each mole of labile phosphate and for each equivalent of aldose liberated upon mild acid hydrolysis. Lowry has investigated the lability of ribose-l-phosphate in dilute hydrochloric acid at room temperature and found that 50% of the ester was split after 2.5 minutes incubation in N hydrochloric acid. In view of these properties and the resynthesis experiments described below the new ester was named ribose-l-phosphate.

The next step was an attempt to resynthesize purine nucleosides with ribose-lphosphate. This was performed by incubating hypoxanthine, ribose-l-phosphate and a fractionated sample of liver nucleosidase about 20 minutes at 25° and subsequently analysing free and incorporated hypoxanthine<sup>5</sup>. It was then found that a large proportion of the hypoxanthine was incorporated in ribosidic linkage and an equimolar amount of labile phosphate was liberated. This enzymatic synthesis of inosine (ribose-l-hypoxanthine) proceeded very far; thus, if equimolar amounts of hypoxanthine and ribosel-phosphate were incubated with the enzyme about 80% of the phosphoriboside was converted into purine-riboside. If the mixture contained twice as much phosphoriboside as hypoxanthine more than 95% of the latter was incorporated in ribosidic linkage. The equilibrium can be formulated as follows: ribose-l-phosphate + hypoxanthine ≥ ribose-l-hypoxanthine + phosphate. The enzyme catalysing this equilibrium was named nucleoside phosphorylase. Nucleoside phosphorylase possesses a certain specificity with regard to the nitrogenous bases added as well as to the pentoses present. Inosine and guanine riboside are the only ribosides which undergo phosphorolysis in the presence of the enzyme used. Adenosine and xanthosine are inert in this system as are pyrimidine ribosides. Likewise hypoxanthine and guanine are the only nitrogenous bases which are incorporated, i.e., which in the presence of the enzyme undergo an exchange with the l-phospho group in ribosc-l-phosphate. This selective trait with regard to purines will be discussed a little later. With regard to the sugar component the furanoid structure of the sugar seems to be imperative for the reaction. Thus, pyranose-ribose-l-phosphate (synthesized by chemical means by Todd and Lythgoe) was practically inactive in the enzyme test as was α-glucose-l-phosphate. Although the furanoid structure of the pentose seems to be essential, other changes in the sugar molecule seem to affect the enzymatic exchange much less. Klein had already observed that liver and spleen nucleosidase catalyse the splitting of purine desoxyribosides just as well as purine ribosides. We have found too that nucleoside phosphorylases fractionated by various means catalyse the phosphorolysis of purine desoxyribosides as well as the purine riboside<sup>6,7</sup>. If we assume that the enzymatic catalysis of the two types of nucleosides is due to the same enzyme and there is good evidence for such an assumption, the substitution of an OH group by a H at carbon no. 2 seems to be unessential for the activity of the liver nucleoside phosphorylase.

#### ENZYMATIC SYNTHESIS OF DESOXYRIBO-NUCLEOSIDES

It was tempting to analyse a little more closely the phosphorolysis of desoxyribosides, and if possible perform an enzymatic synthesis of nucleosides belonging to the desoxyribose series. Friedkin who joined our group here in Copenhagen as a research visitor participated in this project and undertook a closer analysis of some of the components of the system. Guanine desoxyriboside was isolated and subjected to an enzymatic phosphorolysis analogous to that used for ribosides. After removal of the inorganic phosphate the Lowry-Lopez phosphate analysis was performed in order to disclose the the presence of a highly acid-labile ester. The outcome was entirely negative. The failure to detect any ester formation by this method could be due to the fact that the l-ester formed in this case was more stable than ribose-l-phosphate. The other alternative was that the l-ester was even more acid-labile than ribose-l-phosphate. We were inclined towards the latter possibility. This turned out to be correct. If free phosphate and ester phosphate are estimated separately, using precipitation of the true inorganic phosphate by means of ammoniacal ammonium-magnesium sulphate it is possible to detect the formation of a desoxyribose phosphoric ester. This new ester was found to undergo rapid hydrolysis in an acetate buffer of pH 4 at room temperature. FRIEDKIN found that 50% of the desoxyribose phosphate ester was split in 11 minutes at  $25^{\circ}$  at  $p_H$  4. This is presumably the most acid-labile phosphoric ester yet described. It has been possible to show that this ester can act as a precursor for desoxynucleoside synthesis in vitro. The quantitative assay of the desoxyribose ester is under preparation and it can therefore only be stated that if hypoxanthine is incubated with liver nucleoside phosphorylase in the presence of a moderate excess of the desoxyribose ester (but no inorganic phosphate) more than 50% of the hypoxanthine is incorporated with the desoxysugar. The enzymatic formation of a desoxynucleoside was further substantiated by Hoff-JORGENSEN using the microbiological technique<sup>6, 8</sup>. A proper estimation of the amount of aldose present before and after mild hydrolysis of the new desoxyribose ester is under preparation. It is felt most likely that the new ester is an analogue of ribose-l-phosphate, i.e., a desoxyribose-l-phosphate.

Recently Manson and Lampen<sup>9</sup> in Cori's department have prepared an enzyme from thymus gland which brings about a splitting of hypoxanthine desoxyriboside References p. 237.

provided that either phosphate or arsenate is present. The ester formed was isolated and identified as desoxyribose-5-phosphate. The authors have evidence for the presence of an enzyme which catalyses the conversion of a primarily formed l-ester into the 5-ester. The same two authors have also made recent contributions towards our understanding of the enzymatic splitting of pyrimidine desoxynucleosides, especially thymidine<sup>10</sup>. They have isolated an enzyme from bone marrow and kidney which catalyses a splitting of thymine from thymidine, again provided that either phosphate or arsenate is present. The enzyme preparations contain both purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase. Manson and Lampen's observations point also towards a formation of a desoxyribose-l-ester from pyrimidine desoxynucleoside. Thus, addition of hypoxanthine enhances the liberation of thymine from thymidine in the presence of mixed phosphorylases. This effect indicates at least that an enzymatic exchange between hypoxanthine and thymine takes place. However, since the incorporation into ribosidic linkage of hypoxanthine and that of thymine is catalysed by two different enzymes the assumption of a formation of l-phospho-desoxyribose as a common substrate for both enzymes can explain the above mentioned effect.

## THE BIOLOGICAL PATHWAY OF PURINE AND PYRIMIDINE INCORPORATION INTO NUCLEIC ACIDS

The pathway of purine and pyrimidine incorporation into nucleic acids is a problem of major biological importance. The isotope technique has made it possible to make an account of the most significant steps of such a synthesis in the intact organism. In 1941 Schoenheimer and his colleagues initiated some studies on purine incorporation in the intact adult organism. I shall not go into a discussion of the interesting feeding experiments using N15 labelled ammonia and C13 or C14 labelled carbon dioxide which have shed so much light on the synthesis of the purine bases. This discussion is dealing with results of feeding experiments with labelled purines. These studies were initiated by PLENTL AND SCHOENHEIMER<sup>11</sup> and brought into a very successful and fruitful development by the studies performed at the Sloan-Kettering Institute by Brown and coworkers. It will be recalled that PLENTL AND SCHOENHEIMER found that adult rats fed  $N^{15}$  labelled guanine excreted the entire amount of this substance as uric acid and allantoin and correspondingly the guanine of the nucleic acids was found to be devoid of any excess N15. This finding was substantiated 6 to 7 years later by Brown and coworkers. Brown and his colleagues synthesized N15 adenine and guanine according to recent methods developed by Todd and Lythgoe. The most remarkable result of their studies, was the fact that N15 labelled adenine was readily incorporated into the ribonucleic acids both as adenine and guanine<sup>12</sup>. If a moderate amount of N<sup>15</sup> adenine was administered to adult rats about 50% was incorporated as nucleic acid adenine and guanine and the other 50% appeared as allantoin. Bendich and Brown<sup>13</sup> have recently made the interesting observation that 2-6 diamino purine labelled with  $N^{15}$  appears in large amounts in the nucleic acid guanine but not in the adenine. Hypoxanthine seems to be converted exclusively into uric acid and allantoin<sup>14</sup>.

How are the present results of the studies on liver nucleoside phosphorylase to be interpreted in the light of recent findings gained from isotope experiments performed on intact organisms? It will be recalled that the liver nucleoside phosphorylase catalyses the incorporation of only two purine bases, hypoxanthine and guanine — exactly the

two purines bases which according to the studies on the intact organism are not incorporated into the nucleic acids. We are forced to conclude therefore that the type of incorporation of purines which can be demonstrated in incubates with liver enzymes does not represent the final way by which the intact organism incorporates purines for the maintenance of its protoplasmic nucleic acids. It is even justified to question whether the nucleoside phosphorylase has anything whatever to do with the incorporation of purines into nucleic acids. The nucleoside phosphorylase might for instance play a role in processes other than the incorporation of purines into nucleic acids. This brings us to recall the situation with respect to the amino acid oxidases around 1936. At that time Krebs described a water soluble oxidase which catalysed the oxidation of the d-amino acids and which Warburg and Christian purified and identified as a flavine enzyme. Six to seven years later Green, Ratner, and Nocito isolated the oxidase which catalvsed the oxidation of l-amino acids and this also proved to be flavoprotein. When we talk about protein metabolism especially combustion of proteins in the animal organism we realize that the oxidation of the amino acids from proteins must be catalysed by the l-amino acid oxidase and not by the d-amino acid oxidase. The physiological function of the latter enzyme still remains obscure. We may apply the same point of view towards the nucleoside phosphorylase. It appears unlikely that the enzyme should simply serve in the breakdown of purine compounds since, as mentioned earlier, in an enzymatic mixture of free purine, phosphate, nucleoside and phospho-riboside the equilibrium is definitely favourable towards nucleoside formation. The possibility should not be overlooked that formation of inosine from ribose-l-phosphate catalysed by liver nucleoside phosphorylase might represent a primary step in the synthesis of purine ribosides prior to the incorporation of adenine. Adenine might then be exchanged directly with the hypoxanthine present in inosine by an enzyme which does not occur in our usual enzyme preparations. The catalytical action of inosine on the deamination of adenine by a bacterial enzyme<sup>15</sup> might be explained on this assumption; in vitro studies with labelled carbon or nitrogen in the adenine ring should be able to clarify this problem. As regard to the incorporation of pyrimidine into nucleic acid little is known. The recent team work between Bergstrom and Hammarsten and his group<sup>16</sup> has shed interesting light on this problem. It was found that N15 labelled orotic acid can be used as a precursor of the ribonucleic acid pyrimidines of the adult rat. The question regarding incorporation of purines and pyrimidines into desoxyribonucleic acids brings up important new problems regarding the rejuvenation of nuclear components. It is known from the studies by Brues, Tracy, and Cohn and as well as by Hammarsten and Hevesy that the phosphorus in the desoxyribonucleic acids is renewed at a much slower rate than that incorporated in ribonucleic acids. In regenerating or growing tissues the renewal of desoxynucleic acid phosphorus is increased markedly. Likewise Brown and coworkers<sup>17</sup> found that the rate of incorporation of N<sup>15</sup> adenine into desoxyribonucleic acid in the adult rat is negligible as compared with the corresponding processes taking place in the ribonucleic acid. These observations indicating a very slow turnover of desoxyribonucleic acid components in the adult organism coupled with the knowledge of the existence of a highly active desoxynucleoside phosphorylase poses several new questions. For example the enzymatic system catalysing degradation and synthesis of desoxynucleosides in liver should be taken into account in considering the regulatory mechanisms which control transitions between resting and growing states.

As concluding remarks I should like to add that the two types of approaches, the *References p. 237*.

study of enzymatic step reactions in vitro and the study with isotope labelled precursors in vivo are equally indispensable and exert a mutual and valuable influence on each other. An example is the importance of the Embden-Meyerhof glycolysis scheme for the interpretation of the distribution of labelled carbon in glycogen from rats fed with labelled carbon dioxide. The ingenious analysis by Wood and coworkers in this field may well serve as an encouragement for investigators working in allied fields.

#### SUMMARY

The mechanism of incorporation of purines and pyrimidines into ribosidic linkage has been discussed from various points of view. Results gained from enzymatic studies are not in direct agreement with observations made in intact organism using isotopes. Various ways of interpretations are discussed.

#### RÉSUMÉ

Le mécanisme de l'incorporation de purines et de pyrimidines dans la liaison ribosidique a été discuté de différents points de vue. Les résultats obtenus par des études enzymatiques ne concordent pas entièrement avec les observations faites dans l'organisme intact au moyen d'isotopes. Différentes possibilités d'interprétation ont été envisagées.

#### ZUSAMMENFASSUNG

Der Mechanismus der Einverleibung von Purinen und Pyrimidinen in die Ribosid-Bindung ist von verschiedenen Gesichtspunkten aus erörtert worden. Die aus enzymatischen Untersuchungen gewonnenen Ergebnisse stimmen nicht völlig überein mit Beobachtungen welche im unversehrten Organismus mittels Isotopen gemacht wurden. Verschiedene Erklärungsmöglichkeiten werden besprochen.

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### L'ÉNERGIE DE FORMATION DES COMPLEXES DISSOCIABLES ENZYME-SUBSTRAT ET ANTIGÈNE-ANTICORPS

par

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#### I. LES COMPLEXES ENZYME-SUBSTRAT

La connaissance des énergies et entropies de formation des complexes protéiques dissociables permettrait de préciser la nature des liaisons qui y sont impliquées, et de comprendre l'effet spécifique qui en résulte.

Ainsi l'action catalytique des enzymes est généralement expliquée par une attraction entre l'enzyme et le substrat. Plusieurs mécanismes de détail ont été proposés¹. Par exemple, l'attraction de deux substrats juxtaposés sur l'enzyme les presse l'un contre l'autre et favorise leur union. Dans une représentation plus élaborée de l'activation, Stearn² considère l'hydrolyse d'une liaison peptide. La formation du groupe d'atomes activé CONH serait facilitée par l'approche d'un dipole de l'enzyme qui attire l'oxhydrile vers le groupe C-N. L'attraction du substrat par l'enzyme servirait à vaincre les forces de répulsion qui s'opposent à ce rapprochement.

D'autre part, on peut admettre que l'abaissement de l'énergie d'activation ne dépend pas directement de la combinaison de l'enzyme avec le substrat, pris comme un tout et dans son état normal. L'énergie potentielle de l'état activé serait abaissée par résonance d'un groupe réactif du substrat dans l'état activé avec un groupe correspondant de l'enzyme. Le mécanisme suggéré par Delbrück³ pour expliquer l'autoreproduction des protéines s'apparente à cette manière de voir. La connaissance exacte des énergies de liaison serait utile pour entreprendre une discussion serrée de ces deux conceptions.

Or, on ne possède pas de données certaines sur les énergies d'association des enzymes avec leur substrats. Celles dont on dispose jusqu'ici ont été obtenues en appliquant la loi de Van 't Hoff à la variation de la constante de Michaelis en fonction de la température. Comme l'ont mis en évidence Briggs et Haldane¹, cette constante  $K_M$  n'est pas nécessairement égale à l'inverse de la constante d'affinité K de l'enzyme pour son substrat. La variation de  $K_M$  avec la température ne peut donc servir sans réserves à calculer la chaleur de formation à pression constante ou enthalpie  $\Delta$  H du composé. La condition est que la vitesse  $k_1$  de la dissociation du composé ES en E et S, soit grande par rapport à la vitesse  $k_2$  de décomposition du complexe en produit final de la réaction, ou que la décomposition du complexe ait la même énergie d'activation que sa dissociation.

La constante d'affinité K de l'enzyme pour son substrat a bien été déterminée directement, dans une circonstance, par Chance<sup>4</sup>. Elle est 100 fois plus grande que  $1/K_M$ . Il

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s'agit de la peroxydase et du peroxyde d'hydrogène dont l'union donne un composé caractérisé par son spectre d'absorption. Malheureusement la variation de la constante avec la température n'a pas été déterminée, si bien que même dans ce cas on n'a pas encore l'enthalpie. La technique employée par Chance est d'ailleurs restreinte aux associations enzyme-substrat qui ont un spectre d'absorption caractéristique.

Une autre technique, applicable spécialement aux associations des enzymes avec de grosses molécules, peut être fondée sur une autre propriété. On sait mesurer, en principe, les poids moléculaires à partir de l'intensité de la lumière diffusée et tirer des indications sur les dimensions des molécules à partir de la distribution angulaire de cette intensité. Cette technique, actuellement mise en œuvre dans notre laboratoire, pourra être appliquée aux complexes formés entre les enzymes protéolytiques et leur substrat.

# II. L'UNION DE L'AGGLUTININE AUX HÉMATIES

# 1. Equilibre de l'agglutination

Pour un autre type de complexes protéiques dissociables, celui formé par un antigène avec un anticorps, une mesure directe de la chaleur dégagée a été effectuée par Boyd et ses collaborateurs<sup>5</sup>. Ces auteurs ont trouvé que la combinaison de l'hémocyanine avec son anticorps chez le cheval, dégage 40 000 calories par molécule d'antihémocyanine.

On a depuis Arrhénius cherché à obtenir la chaleur de réaction à partir de l'effet de la température sur l'équilibre qui s'établit entre antigènes et anticorps. La difficulté est d'expliciter la relation qui unit les constantes d'équilibre à la composition du complexe formé. En particulier, les résultats dépendent de l'idée que l'on se fait de la structure de ce complexe, de la valence des constituants, et des interactions entre les groupes réactifs d'une même molécule.

Nous avons pensé que le procédé statistique le plus simple pouvait être appliqué à l'isohémagglutination. Celle-ci étant une réaction de surface, on devait être à même de calculer, avec un minimum d'hypothèses, la relation existant entre la grandeur observée et une constante d'équilibre. Ce phénomène présentait en outre l'avantage que sa réversibilité avait été très sûrement prouvée.

Soit  $\tau$  le taux d'a glutination, c'est-à-dire, en appelant  $N_1$  le nombre d'hématies libres, le rapport entre le nombre des hématies agglutinées  $(N_o-N_l)$  et le nombre total d'hématies  $N_o$ . Filitti-Wurmser et Jacquot-Armand ont établi que, par numération dans un hématimètre, l'erreur standard sur le taux d'agglutination varie entre 0.3% pour  $\tau=0.99$  et 7% pour  $\tau=0.45$ . La technique est donc utilisable pour une étude quantitative. Elle a servi à démontrer la réversibilité de l'agglutination par les faits suivants.

a) Dissociation de l'agglutinat. On obtient le même état d'équilibre quand on agglutine des hématies ou quand on dissocie un agglutinat.

Pour le prouver on mélange dans une première opération un sérum avec un nombre donné d'hématies et une solution tampon de manière à avoir un volume V. On obtient un certain taux d'agglutination. Dans une deuxième opération on mélange le même sérum avec le même nombre d'hématies et une quantité de solution tampon telle que le volume v est plus petit que V. Il se forme un agglutinat plus abondant. Lorsque celui-ci a atteint son équilibre, on dilue jusqu'au volume V. Le nouveau taux d'agglutination qui s'établit est égal à celui obtenu dans la première opération.

b) Déplacement de l'équilibre par la température. Lorsque, à un sérum donné, on ajoute des quantités croissantes d'hématies, on obtient, suivant la température à laquelle on

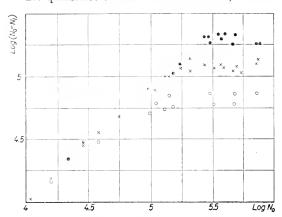


Fig. 1. log (hématies agglutinées) en fonction de log (hématies totales) • à 15° C, × à 25° C, ∘ à 37° C

opère, les résultats représentés par la Fig. 1. Sur ce diagramme on a porté en abscisses log  $N_o$  et en ordonnées log  $(N_o-N_l)$ . On voit que le nombre maximum d'hématies agglutinées augmente quand la température s'abaisse.

En outre, le taux d'agglutination est d'abord voisin de l'unité (portion linéaire des courbes) à toutes les températures, mais au-delà d'une certaine valeur de  $N_o$ , le taux d'agglutination est nettement plus élevé quand la température est plus basse. On peut mettre en évidence le déplacement réversible de l'équilibre par le fait qu'un même taux d'agglutination est atteint soit directe-

ment à 37°, soit après une mise en équilibre à 5° suivie d'une dissociation partielle de l'agglutinat à 37°.

Il fallait, pour l'application de la statistique que nous voulions faire, s'assurer que l'effet de la température n'est pas dû à l'existence de groupes actifs différents. Plusieurs preuves en ont été données: en particulier, de l'agglutinine extraite par élution d'un agglutinat formé à 37° présente le même effet de température que le sérum lui-même.

# 2. Détermination de l'énergie de formation du complexe agglutinine — groupe agglutinogène

Nous avons donc admis<sup>7</sup> que l'agglutination résulte de la fixation de molécules d'agglutinine A sur des groupes G tous pareils situés à la surface des hématies, et assez éloignés les uns des autres pour être sans interactions. Les hématies qui s'agglutinent sont celles qui ont fixé en moyenne un nombre minimum l de molécules d'agglutinine. Il suffit alors pour obtenir le taux d'agglutination en fonction de la concentration d'agglutinine (A) d'appliquer un raisonnement classique.

S'il existe à la surface de chaque hématie m groupes capables de réagir réversiblement avec l'agglutinine, il y aura une distribution des hématies HA portant un nombre n de molécules d'agglutinine, n variant de n0 (hématies nues) à n0 (hématies saturées).

Soit K la constante "intrinsèque" correspondant à l'équilibre:

$$G + A \rightleftharpoons G A$$

entre l'agglutinine et les groupes agglutinogènes supposés réagir comme s'ils étaient des molécules séparées; K(A)/I + K(A) est la probabilité pour qu'un groupe individuel fixe une molécule d'agglutinine. En portant cette valeur dans la relation de Bernoulli, on trouve que le taux d'agglutination est:

$$\tau = [1 + K(A)]^{-m} \sum_{n=1}^{n=m} \frac{m!}{n!(m-n)!} [K(A)]^n$$

La variation du taux d'agglutination en fonction de la concentration d'agglutinine à une température donnée, peut être obtenue expérimentalement. On sait titrer l'agglu-Bibliographie p. 243. tinine en valeurs relatives a (A) d'après le nombre maximum d'hématies agglutinées à  $4^{\circ}$  C. On obtient la courbe  $\tau = f[a(A)]$  de la manière suivante: les valeurs de  $\tau$  sont

déterminées directement dans une première agglutination en comptant les hématies restées libres dans les mélanges constitués par une quantité fixe de sérum et des quantités croissantes d'hématies  $\delta$  dans un volume constant. Les valeurs correspondantes de  $\alpha$  (A) proviennent des titrages effectués par une série d'agglutinations pratiquées cette fois sur le liquide obtenu en centrifugeant chacun des mélanges ayant servi à la mesure de  $\tau$ , après que l'équilibre d'agglutination a été atteint.

Les courbes de la Fig. 2 représentent les résultats obtenus pour des agglutinations d'un même sérum du groupe A, à  $25^{\circ}$  C et à  $37^{\circ}$  C.

On détermine à partir de ces courbes le rapport des valeurs de K à 25°C et à 37°C, en faisant

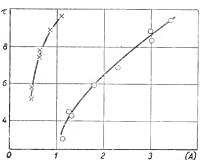


Fig. 2. Taux d'agglutination en fonction de la concentration d'agglutinine non fixée (en valeurs relatives) × à 25° C, ∘ à 37° C

comme seule hypothèse que le nombre l ne varie pas ou varie très peu avec la température. Ce rapport  $K_{25}/K_{37}$  est égal au rapport  $(A)_{37}/(A)_{25}$  des concentrations relatives d'agglutinines pour un même taux d'agglutination. La valeur trouvée est  $3.5 \pm 0.2$ , ce qui correspond à une enthalpie  $\Delta$  H de —19000 calories.

Une détermination de  $\Delta$  H qui n'implique pas d'hypothèse sur le mécanisme de l'agglutination proprement dite, consiste à porter en abscisses des grandeurs proportionnelles à  $\tau/K(A)$  et en ordonnées des grandeurs proportionnelles à  $\tau/A_f$ , en appelant  $A_f$  l'agglutinine fixée divisée par la totalité des hématies. Cette quantité est

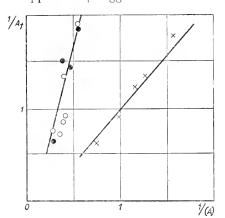


Fig. 3. Inverse de la quantité d'agglutinine fixée (en valeurs relatives) en fonction de l'inverse de la concentration d'agglutinine non fixée (en valeurs relatives). Sérum No. 2519, × à 25° C, • à 37° C; Sérum No. 1028, ° à 37° C

mesurée par différence entre l'agglutinine initiale et l'agglutinine restante. On doit obtenir une droite, si les groupes sont sans interaction:

$$\frac{\mathbf{I}}{\mathbf{A}_{\mathsf{f}}} = \frac{\mathbf{I}}{m} + \frac{1}{2} \frac{\mathbf{I}}{|m\mathbf{K}(\mathbf{A})|} \tag{1}$$

Le rapport des pentes à 2 températures 37° C et 25° C est égal au rapport:  $K_{25}/K_{37}$ .

La Fig. 3 montre les points expérimentaux et les droites calculées<sup>8</sup> d'après la méthode des moindres carrés, pour un même sérum (2519) à deux températures 37° C et 25° C, et pour un autre sérum (1028) à 37° C. Il s'agit de 2 sérums de titre élevé (N<sub>max 4°</sub> est égal à 1254000 par µl pour le sérum 2519 et à 1925000 pour le sérum 1028).

Les pentes correspondantes pour le sérum 2519 sont: à 37° C, 1.851 avec une erreur standard  $\sigma = 0.147$  et à 25° C, 0.573 avec une erreur stan-

dard  $\sigma=0.017$ . Le rapport  $K_{25}/K_{37}$  est donc 3.23 et l'enthalpie  $\varDelta$  H —18000 calories. La concordance avec le résultat précédent —19000 calories est satisfaisante.

### III. DISCUSSION

En ce qui concerne la nature des liaisons, on notera que 20000 calories correspondent à la formation d'environ 4 liaisons hydrogène ou à une vingtaine d'attractions de Van der Waals (Pauling<sup>9</sup>). Ces valeurs sont raisonnables si l'on admet, par exemple, qu'un groupe agglutinogène renferme un polysaccharide.

On peut avoir une idée de la grandeur de la constante d'équilibre K. Cette constante "intrinsèque" caractérise l'équilibre entre l'agglutinine et les groupes agglutinogènes supposés indépendants. Elle est égale à une constante d'équilibre classique entre l'agglutinine et les hématies portant un nombre  $\frac{m+1}{2}$  de groupes combinés, c'est-à-dire les hématies demi-saturées, parce que pour ces hématies l'effet statistique sur l'énergie libre est éliminé.

Nous avons utilisé les données de Kabat<sup>10</sup> sur la concentration de l'isoagglutinine dans les sérums pour calculer la valeur de m à partir du rapport (A)<sub>o</sub>/(A) des concentrations d'agglutinine avant et après agglutination en présence d'un petit nombre d'hématies (environ  $4\cdot 10^3$  par  $\mu$ l). On trouve ainsi que m est de l'ordre de  $10^6$ , qui correspond d'ailleurs sensiblement au maximum de place disponible pour l'agglutinine à la surface d'une hématie. Cette valeur de m portée dans la relation (1) donne alors pour K la valeur  $2\cdot 10^8$  à  $4^\circ$  C, soit  $1\cdot 10^7$  à  $37^\circ$  C.

A cette dernière température la variation d'énergie libre par molécule-gramme d'agglutinine est  $\Delta$  F =  $-10\,000$  calories et la variation d'entropie  $\Delta$  S = -30, environ 8 unités par liaison. Toutes ces valeurs apparaissent vraisemblables.

L'énergie libre ainsi trouvée est à comparer avec la valeur calculée selon les procédés ordinaires de la théorie statistique, par Morales, Botts et Hill¹ pour l'énergie libre de combinaison d'une molécule d'antihémocyanine avec une molécule d'hémocyanine. Ces auteurs partent de la donnée calorimétrique de Boyd et collaborateurs. Ils tiennent seulement compte, pour obtenir la fonction de partition, des effets de translation et de rotation et supposent que les deux molécules ont même masse et même rayon, et que le moment d'inertie du complexe est celui d'une sphère équivalente. Leur résultat -11000 calories par groupe fixé est tout à fait voisin de celui que nous obtenons pour la combinaison de l'agglutinine avec un groupe agglutinogène d'une hématie. Mais dans le cas de l'hémocyanine, l'énergie totale étant de 40000 calories, 8 liaisons de 5000 calories, au lieu de 4, sont impliquées dans la formation du complexe; l'énergie libre par liaison est donc moitié de celle trouvée pour l'agglutinine.

La cohérence des résultats obtenus dans le cas de l'isohémagglutination présente un autre intérêt que celui de donner une base aux hypothèses possibles sur la nature des liaisons en jeu. Il sera utile d'introduire la mesure de ces grandeurs énergétiques dans la comparaison de sérums d'origines diverses. Après un examen plus approfondi des facteurs accessoires (force ionique, présence d'inhibiteurs), susceptibles de les faire varier pour une même agglutinine, il n'est pas exclu qu'il se dégage, d'une telle comparaison, des caractères de groupes intéressants, même à un point de vue strictement biologique.

# RÉSUMÉ

On ne connaît pas de données rigoureuses sur l'énergie de liaison des enzymes à leur substrats. En ce qui concerne l'union des antigènes aux anticorps, il n'existait qu'une détermination calorimétrique de l'union de l'hémocyanine à l'antihémocyanine. L'étude de l'isohémagglutination a permis de calculer l'énergie de la liaison agglutinine-groupe agglutinogène et d'évaluer la constante d'équilibre correspondante, soit  $1 \cdot 10^7$  à  $37^\circ$  C.

### SUMMARY

No exact data are known about the energy of the bonds between enzymes and their substrates. As to the attachment of antigens to antibodies only a calorimetric determination of the bond haemocyanin-antihaemocyanin was known. The study of isohaemagglutination has permitted the calculation of the bond-energy of the complex agglutinin-agglutinogenic group and the estimation of the corresponding equilibrium constant, being 1·107 at 37° C.

### ZUSAMMENFASSUNG

Man kennt keine genauen Angaben über die Bindungsenergie der Enzyme an ihre Substrate. Was den Komplex Antigen-Antikörper anbelangt, so ist nur eine kalorimetrische Bestimmung der Bindung von Haemocyanin an Antihaemocyanin bekannt.

Die Untersuchung der Isohaemagglutination erlaubt die Energie der Verbindung Agglutininagglutinogene Gruppe zu berechnen und die entsprechende Gleichgewichtskonstante, 1·107 bei 37° C, anzugeben.

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# NÉCESSITÉ D'UN COENZYME POUR LE FONCTIONNEMENT DE LA DÉSULFINICASE

pa:

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L'action de divers extraits de foie sur l'acide cystéinesulfinique¹ est susceptible de présenter des irrégularités notables, quoique ces extraits aient été obtenus dans des conditions apparemment identiques. Recherchant la cause de ces irrégularités, nous avons constaté qu'elles sont dues, au moins en partie, à une perte plus ou moins importante en un facteur indispensable au fonctionnement de la désulfinicase, perte qui a lieu au cours de la préparation de l'enzyme. Ce facteur est un coenzyme dont nous ignorons encore la nature; nous savons seulement qu'il est constitué par une molécule organique et que, en dehors du foie, il existe également dans la levure. Dans le présent travail, nous donnons quelques résultats expérimentaux qui mettent en évidence l'importance de ce coenzyme dans la désulfination enzymatique de l'acide cystéinesulfinique.

### PARTIE EXPÉRIMENTALE

La solution de désulfinicase est obtenue en traitant pendant 30 minutes à  $0^{\circ}$ , n g de poudre acétonique de foie de lapin<sup>2</sup> par n. 10 ml d'eau distillée. On centrifuge, lave le culot de centrifugation avec un peu d'eau qu'on ajoute à la solution enzymatique, et on complète le volume à n. 10 ml avec de l'eau. Le poids sec d'un tel extrait est de l'ordre de 30 mg par ml.

Les solutions de coenzyme débarrassées d'apoenzyme sont obtenues en traitant au bain-marie bouillant pendant 4 minutes la solution enzymatique précédente. On élimine par centrifugation les protéines coagulées par la chaleur, et on concentre sous vide, de telle sorte que 10 ml d'une telle préparation corresponde à un poids donné de la poudre acétonique extraite initialement. Le poids sec d'une solution de coenzyme contenant l'extrait de 1 g de poudre acétonique de foie dans 10 ml est de l'ordre de 10 à 15 mg par ml.

Les tubes utilisés dans les expériences et la mesure de l'activité des systèmes enzymatiques ont été décrits antérieurement. Les expériences sont faites ici en solution de bicarbonate de sodium à 0.16% et sous atmosphère d'azote contenant 10% d'anhydride carbonique; le p<sub>H</sub> du milieu est ainsi de 7.3. La température est de 35°, et la durée d'action est de 2 heures. Les résultats sont exprimés en micromolécules d'anhydride sulfureux dégagé.

1. Séparation du coenzyme par dialyse et réactivation de l'apoenzyme par addition de coenzyme

Dans 5 tubes contenant chacun 130 micromolécules de cystéinesulfinate de sodium dans 10 ml de solution de bicarbonate de sodium à 0.16%, on introduit:

Tube I: 10 ml de solution d'enzyme additionnée de bicarbonate de sodium à 0.16%, et correspondant à 1 g de poudre acétonique; cette solution est préparée extemporanément. Plus 5 ml de solution de bicarbonate.

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Tube II: 10 ml de solution d'enzyme analogue à la précédente; mais cette solution a été préalablement maintenue pendant 7 heures à 0°. Plus 5 ml de solution de bicarbonate.

Tube III: 10 ml de solution d'enzyme analogue aux précédentes; mais cette solution a été préalablement dialysée pendant 7 heures à 0° contre une solution de bicarbonate de sodium à 0.16%. Plus 5 ml de solution de bicarbonate.

Tube IV: 10 ml de la solution d'enzyme dialysée comme dans le tube précédent, plus 5 ml d'une solution de coenzyme correspondant à 1 g de poudre acétonique, additionnée de bicarbonate de sodium à 0.16%.

Tube V: 5 ml de la solution de coenzyme utilisée dans le tube précédent, plus 10 ml de solution de bicarbonate.

Les résultats obtenus sont donnés dans le Tableau I.

TABLEAU I
INACTIVATION ET RÉACTIVATION DE LA DÉSULFINICASE
PAR ÉLIMINATION, PUIS ADDITION DE COENZYME

| Tube  | $\mathrm{SO}_2$ dégagé | Tube    | $\mathrm{SO}_{2}$ dégagé |
|-------|------------------------|---------|--------------------------|
| I III | 40<br>29<br>5          | IV<br>V | 30<br>6                  |

Les chiffres du Tableau I montrent que: 1. la désulfinicase perd son activité par dialyse; 2. son activité réapparait après addition d'une solution de coenzyme; 3. la solution de coenzyme ne présente elle-même qu'une très faible activité; 4. le maintien de l'enzyme pendant 7 heures à 0° provoque une certaine inactivation.

# 2. Activation par le coenzyme d'un extrait non dialysé

Les solutions de désulfinicase obtenues par la méthode utilisée ici donnent normalement, sans addition supplémentaire de coenzyme, un dégagement d'anhydride sulfureux de 50 à 55  $\mu$ mol après 2 heures, pour 10 ml de solution enzymatique agissant sur 130  $\mu$ mol de cystéinesulfinate de sodium dans les conditions décrites; exceptionnellement, on obtient des préparations plus actives; mais on rencontre assez souvent des préparations fermentaires qui présentent une activité plus faible. Ces diverses préparations peuvent être généralement activées par addition de coenzyme. En voici un exemple:

Dans 5 tubes contenant chacun 130 micromolécules de cystéinesulfinate de sodium dans 10 ml de solution de bicarbonate de sodium à 0.32% et 10 ml de solution de désulfinicase, on introduit 5 ml de solution de coenzyme en concentrations croissantes, additionnées de bicarbonate à 0.16%. Dans un sixième tube, les 10 ml de solution de désulfinicase sont remplacés par 10 ml de solution de bicarbonate.

Le Tableau II présente les résultats obtenus.

Ces résultats montrent que l'addition de coenzyme donne à la préparation enzymatique une activité maximum qui ne peut être ensuite dépassée, quelle que soit la quantité de coenzyme ajoutée en excès.

### TABLEAU II

ACTIVATION PAR LE COENZYME D'UNE SOLUTION DE DÉSULFINICASE

La concentration de la solution de coenzyme représentée par 1 est telle que 10 ml de solution correspondent à 1 g de poudre acétonique.

| т. ь  | Solution de désulfinicase | Concentration de la solution  | $\mathrm{SO}_2$ dégagé |          |  |  |
|-------|---------------------------|-------------------------------|------------------------|----------|--|--|
| Tube  | (ml)                      | de ta solution<br>de coenzyme | absolu                 | corrigé* |  |  |
| I     | 10                        | О                             | 31                     | 31       |  |  |
| $\Pi$ | 10                        | 1                             | 5 I                    | 45       |  |  |
| HH    | 10                        | 2                             | 65                     | 5-4      |  |  |
| 1 V   | 10                        | 4                             | 75                     | 53       |  |  |
| V     | 10                        | 6                             | 88                     | 55       |  |  |
| VI    | O                         | 2                             | ΙI                     |          |  |  |
|       |                           |                               |                        |          |  |  |

<sup>\*</sup> Corrections tenant compte de l'activité résiduelle des solutions de coenzyme; les chiffres corrigés représentent l'activité propre de la solution de désulfinicase réactivée.

# 3. Stabilité du coenzyme à la chaleur

Les expériences précédentes indiquent que les solutions de coenzyme obtenues après un chauffage de 4 minutes présentent encore par elles-mêmes une légère action sur l'acide cystéinesulfinique. Nous avons constaté qu'il est possible de supprimer pratiquement cette action en traitant la solution enzymatique au bain-marie bouillant pendant 15 minutes au lieu de 4. Mais on obtient alors des solutions de coenzyme sensiblement moins actives. L'expérience présentée ici est faite dans les conditions suivantes:

Dans 5 tubes contenant chacun 65 micromolécules de cystéinesulfinate de sodium dans 10 ml de solution de bicarbonate de sodium à 0.32%, on introduit soit 5 ml de solution de désulfinicase et 5 ml d'eau (S), soit 5 ml de solution de désulfinicase et 5 ml de solution de coenzyme, cette dernière correspondant à l'extraction de 2 g de poudre acétonique (SC), soit 5 ml de solution de coenzyme et 5 ml d'eau (C). Les résultats obtenus sont fournis par le Tableau III.

TABLEAU III
INFLUENCE DU TEMPS DE CHAUFFAGE SUR LE COENZYME

| Contenu<br>des tubes | Temps au   | $\mathrm{SO}_2$ dégagé |         |  |  |  |
|----------------------|------------|------------------------|---------|--|--|--|
|                      | bain-marie | absolu                 | corrigé |  |  |  |
| S                    | _          | 15                     | 15      |  |  |  |
| SC                   | 4          | 38                     | 32      |  |  |  |
| SC                   | 15         | 27                     | 25      |  |  |  |
| С                    | 4          | 6                      | _       |  |  |  |
| C                    | 15         | 2                      | -       |  |  |  |

<sup>\*</sup> Voir note du Tableau II.

# 4. Mise en évidence de la nature organique du coenzyme

Les cendres de la solution de coenzyme sont incapables d'activer l'apoenzyme de la désulfinicase. L'expérience est faite ici avec une solution de désulfinicase non préalablement dialysée, mais susceptible toutefois d'avoir son activité notablement accrue par addition de coenzyme. Les cendres sont obtenues par calcination dans une capsule de

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platine de l'extrait sec de 10 ml de solution de coenzyme correspondant à 4 g de poudre acétonique. Le produit de cette calcination est dissous dans l'eau acidulée et la solution, ajustée à p<sub>H</sub> 7.0 est ramenée à 10 ml. Chaque tube contient 65 micromolécules de cystéinesulfinate de sodium dans 10 ml de solution de bicarbonate de sodium à 0.32%. Les tubes sont additionnés en outre de soit 5 ml de solution de désulfinicase et 5 ml d'eau (S), soit 5 ml de solution de désulfinicase et 5 ml de solution de coenzyme (SC), soit 5 ml de solution de désulfinicase et 5 ml de solution de cendres (SM). Les poids secs, en mg par ml, des diverses solutions, sont les suivants: désulfinicase 30, coenzyme 38, cendres 6.5.

Le Tableau IV indique les résultats obtenus.

TABLEAU IV actions comparées du coenzyme et de ses cendres

| Contenu des tubes | SO <sub>2</sub> dégagé |
|-------------------|------------------------|
| S                 | 24                     |
| SC                | <b>5</b> 8             |
| SM                | 2                      |



Il apparaît ainsi que, non seulement les cendres n'ont aucun pouvoir activant vis-à-vis de la désulfinicase, mais que, au contraire, elles exercent une action inhibitrice nette. Le mécanisme de cette action est actuellement à l'étude.

# 5. Action de divers coenzymes sur la désulfinicase

La nature organique d'une partie au moins du coenzyme de la désulfinicase ayant été établie, il était intéressant de rechercher si des coenzymes connus étaient capables d'activer l'apoenzyme de la désulfinicase. Parmi ces coenzymes, deux sont particulièrement intéressants par suite de l'analogie des réactions auxquelles ils participent, réactions de décarboxylation, avec la désulfination de l'acide cystéinesulfinique, ce sont la cocarboxylase et le phosphate de pyridoxal. Sans qu'il soit utile de donner ici de chiffres, disons que à la dose de 500 µg par tube (20 ml), et en présence ou en absence de 1 mg de chlorure de magnesium, aucune activation de la désulfinicase n'a pu être mise en évidence avec les substances suivantes: cocarboxylase, phosphate de pyridoxal, pantothénate de calcium, lactoflavine. Il apparaît a priori peu probable que le phosphate de lactoflavine et les codéhydrogénases, que nous n'avons pas encore essayés, aient ici une action. Il semble donc que la codésulfinicase diffère des coenzymes actuellement connus.

# 6. Présence de la codésulfinicase dans la levure

On traite de la levure de boulangerie en la chauffant avec son poids d'eau à 100° pendant 15 minutes; le liquide obtenu après centrifugation contient le coenzyme, comme le montre l'expérience suivante:

Dans 3 tubes contenant chacun 65 micromolécules de cystéinesulfinate de sodium dans 10 ml de solution tampon de phosphates à p<sub>H</sub> 7.35, on introduit:

Tube I: 5 ml de solution de désulfinicase moyennement active, plus 5 ml d'eau.

Tube II: 5 ml de la solution précédente de désulfinicase, plus 5 ml du liquide d'extraction de la levure.

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Tube III: 5 ml du liquide d'extraction de la levure, plus 5 ml d'eau.

Les quantités d'anhydride sulfureux dégagé après 2 heures à  $35^{\circ}$  en atmosphère d'azote, sont données dans le Tableau V.

L'activation par l'extrait de levure, qui n'exerce lui-même aucune action sur l'acide cystéinesulfinique, est très nette.

Nous sommes heureux de remercier ici MM. Gunsalus et Westenbrink qui nous ont aimablement envoyé les échantillons de phosphate de pyridoxal et de cocarboxylase utilisés ici.

TABLEAU V
ACTIVATION DE LA DÉSULFINICASE PAR UN EXTRAIT DE LEVURE

| Tube      | $\mathrm{SO}_2$ dégagé |
|-----------|------------------------|
| III<br>II | 16<br>47<br>0          |

# RÉSUMÉ

La désulfinicase, inactivée par dialyse, récupère son activité après addition d'un extrait de foie incapable par lui-même d'agir sur l'acide cystéinesulfinique. L'activité des solutions de désulfinicase, même non dialysées, est généralement accrue par addition d'extrait de foie ou d'extrait de levure. Il apparaît ainsi que la désulfinicase nécessite pour son fonctionnement la présence d'un coenzyme, facilement dissociable de l'apoenzyme. Ce coenzyme est de nature organique et diffère de la cocarboxylase et du phosphate de pyridoxal.

### SUMMARY

Desulphinicase, inactivated by dialysis, regains its activity after addition of a liver extract which itself is incapable of acting on cysteine-sulphinic acid. The activity of desulphinicase solutions, also undialysed ones, is generally increased by addition of liver extract or yeast extract. It thus appears that desulphinicase necessitates for its functioning the presence of a coenzyme, readily dissociable from the apoenzyme. This coenzyme is organic in nature and differs from cocarboxylase and pyridoxal phosphate.

### ZUSAMMENFASSUNG

Durch Dialyse inaktivierte Desulfinicase erlangt ihre Wirksamkeit wieder nach Beifügung eines Leberextraktes der für sich selbst unfähig ist, auf Cysteinsulfiniaure einzuwirken. Die Wirksamkeit von Desulfinicase-Lösungen, sogar undialysierten, wird im Allgemeinen durch Zusatz von Leber- oder Hefeextrakten verstärkt. Es scheint also, dass die Desulfinicase zu ihrer Wirkung ein Coenzym braucht, welches leicht vom Apoenzym dissozierbar ist. Dieses Coenzym ist organischer Natur, jedoch verschieden von Cocarboxylase und von Pyridoxalphosphat.

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# BODY SIZE AND TISSUE RESPIRATION

by

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It has long been known that in homoiothermic animals the basal metabolic rate, per unit of body weight, decreases with the size of the animal, and the question has often been discussed whether the respiration of individual tissues of animals of different size shows the same differences as the intact organisms. Terroine and Roche¹ and Grafe, Reinwein, and Singer² measured the respiration of various tissues in vitro and came to the conclusion that homologous tissues of different animals respire in vitro at about the same rate, irrespective of the size of the animal. They ascribed the differences found in the intact animal to the regulatory influences of the nervous system and of hormones. Kleiber³, ⁴ on the other hand, reported that the rate of respiration of liver slices of rats, rabbits, sheep, horses and cows, per unit of weight, decreased with increasing size of the animal. The decrease observed was of the same order as the decrease of the basal metabolism of the living animal.

This lack of agreement is not due to discrepancies in experimental observations but arises from difficulties of procedure and interpretation. Whilst the measurement of the basal metabolic rate is a standardized technique, no accepted standards exist for the measurement of the oxygen uptake of isolated tissues *in vitro*. It has often been demonstrated that the oxygen uptake of tissues *in vitro* is not a constant value. Specimens of the same tissue can show wide and reproducible variations, depending on the conditions under which the measurements are made. Among the factors responsible for these variations two are of special importance: the composition of the medium in which the tissue is suspended and the physical treatment of the material. As the part played by these factors was not fully appreciated in previous investigations it was thought that new measurements of the rate of respiration of isolated tissues under standard conditions are needed. As a preliminary it was necessary to define standard conditions which would resemble as closely as possible the state of the tissues in the intact, possibly resting, animal, and which would yield a "standard rate" of tissue respiration.

A. GENERAL CONSIDERATIONS CONCERNING THE MEASUREMENT OF THE
"STANDARD RATE" OF TISSUE RESPIRATION

### 1. Treatment of tissue

In order to measure the rates of metabolic processes in isolated tissues it is, as a rule, unavoidable to subject the tissues to procedures like slicing, mincing or homogenizing, so that the cells can be satisfactorily supplied with oxygen and substrates. References p. 267–269.

These procedures affect different tissues in different ways. In the case of kidney cortex, liver, striated, and cardiac muscle, mince and homogenates show higher initial rates of respiration than sliced material when phosphate saline without a combustible substrate is used as the medium. If the medium contains substrates which stimulate respiration of slices, such as lactate, pyruvate, fumarate, and glutamate, homogenates, mince and slices give approximately the same rates of respiration<sup>5, 6, 7</sup>. In these tissues minced or homogenized materials give the maximum rate of respiration. In other tissues, e.g., spleen<sup>8</sup>, and lung<sup>9</sup>, minced and homogenized material gives consistently lower rates of respiration than sliced material. The low values have been attributed to the hydrolysis of coenzymes by nucleosidases released on the destruction of the tissue<sup>9</sup>.

It is reasonable to assume that slicing leaves the tissue nearer to the 'natural' state than mincing or homogenizing, because the number of physically damaged cells is bound to be much smaller in slices than in mince or homogenates. Slicing is therefore suggested as the procedure of choice for the measurement of the standard rate of metabolism.

# 2. Choice of medium

In this section 5 different media are considered for the measurement of a standard rate of respiration. They are:

Serum

Supplemented serum

Saline serum substitute (later referred to as 'medium I')

Phosphate saline without Ca, low in bicarbonate and  $CO_2$  (later referred to as 'medium II')

Saline low in phosphate, bicarbonate, and CO<sub>2</sub> (later referred to as 'medium III').

Scrum. Plasma or serum, being the natural environment of animal tissues, suggest themselves as the most physiological standard media. Plasma requires the addition of an anticoagulant and several of these, e.g., sodium fluoride and sodium oxalate, are unsuitable as they inhibit metabolic processes. Among the remaining substances heparine is least likely to affect tissue metabolism, but relatively large amounts are required to prevent coagulation in the presence of tissues. In general serum is preferable to plasma because the absence of fibrinogen from the medium is less likely to affect the activities of the tissue than the addition of an anticoagulant.

Supplemented serum. Although serum resembles the physiological environment more closely than any other medium it is by no means a perfect medium for in vitro experiments. A tissue suspended in plasma or serum may, by its metabolism, soon cause major changes in the concentration of important constituents, such as glucose, pyruvate, lactate, and the acids of the tricarboxylic cycle, and also of bicarbonate. In the intact body the balance of activities of all organs maintains a relative constancy of the concentration of serum constituents; thus, glucose used up by some tissues, is replenished from liver stores and by the absorption from the gut. But in vitro the metabolic activity of a single tissue can rapidly convert serum into an 'unphysiological' medium by exhausting the available substrates.

Another factor to be taken into consideration is the circumstance that in the intact organ the path of diffusion is much shorter than *in vitro*, the average distance between capillary wall and tissue cell being much shorter than the average distance between the

surface and the centre of the slice. Hence a concentration gradient and a rate of diffusion which might be sufficient to saturate the cells *in vivo* may become a limiting factor *in vitro*.

Both difficulties—rapid exhaustion and slow diffusion—can be overcome by increasing the concentration of the 'relevant' metabolites in the medium. This consideration raises the question of what are 'relevant' substrates. Among the very large number of organic substances known to occur in plasma and serum (listed in Table I) only a few have been found to influence the oxygen uptake *in vitro*. They are glucose, lactate, pyruvate, the acids of the tricarboxylic cycle, and glutamate (or glutamine), and some closely related substances such as phosphorylated intermediates of glycolysis which need not be considered separately. A few special amino acids (*e.g.*, tyrosine, phenylalanine, proline) can increase the respiration of liver, kidney, and spermatozoa<sup>61, 62, 63, 64</sup>, but although these effects may be of importance in relation to the

TABLE I
COMPOSITION OF HUMAN BLOOD PLASMA

|                                     | mg/100                          |                                   |            |
|-------------------------------------|---------------------------------|-----------------------------------|------------|
| Substance                           | Average or representative value | Range or<br>standard<br>deviation | References |
| Nitrogenous substances              |                                 |                                   |            |
| Protein (total)                     | 6720                            |                                   | 10         |
| Albumin                             | 4040                            | S.D. 270                          | 10         |
| $a_1$ -Globulin                     | 310                             | S.D. 51                           | 10         |
| $a_2$ -Globulin                     | 480                             | S.D. 83                           | 10         |
| β-Globulin                          | 810                             | S.D. 126                          | 10         |
| γ-Globulin                          | 740                             | S.D. 151                          | 10         |
| Fibrinogen                          | 340                             | S.D. 59                           | 10         |
| Non-protein nitrogen (total)        | 25                              | 18-30                             | 11         |
| Amino-N (as N, ninhydrin method)    | 4. I                            | 3.4-5.5                           | 12, 13     |
| Amino-N (as N, nitrous acid method) | 4.4                             | 3.7-5.9                           | 12         |
| Alanine                             | 3.97                            | S.D. 0.70                         | 14. 15     |
| Arginine                            | 2.34                            | S.D. 0.62                         | 16, 17, 18 |
| Citrulline                          | 0.50                            | 0.38-0.59                         | 19         |
| Glutamic acid                       | 3.41                            | S.D. 1.39                         | 20         |
| Glutamine                           | 5.78                            | S.D. 1.55                         | 20         |
| Glycine                             | 1.77                            | S.D. 0.26                         | 1.4        |
| Histidine                           | 1.42                            | S.D. 0.18                         | 16, 18     |
| Iso-leucine                         | 1.60                            | S.D. 0.31                         | 16, 18     |
| Leucine                             | 1.91                            | S.D. 0.34                         | 16, 18     |
| Lysine                              | 2.95                            | S.D. 0.42                         | 16, 18     |
| Methionine                          | 0.85                            | 0.46-1.48                         | 21, 18     |
| Phenylalanine                       | 1.38                            | S.D. 0.32                         | 16         |
| Threonine                           | 2.02                            | S.D. 6.45                         | 16, 18     |
| Tryptophane                         | 1.08                            | S.D. 0.21                         | 16, 22     |
| Tyrosine                            | 1.48                            | S.D. 0.37                         | 16         |
| Valine                              | 2.83                            | S.D. 0.34                         | 16, 18     |
| Ammonia (as N, whole blood)         | below 0.05                      |                                   | 23, 24     |
| Creatine                            | 0.9                             | 0.62-1.02                         | 25         |
| Creatinine                          | 0.4                             | 0.28-0.62                         | 25         |
| Glycocyamine                        | 0.26                            | 0.24-0.28                         | 17         |
| Urea (as N)                         | I 2                             | 10-17                             | 11, 26     |
| Uric acid                           | 4                               | 2-6                               | 27         |
| Allantoin                           |                                 | 0.3-0.6                           | 19         |
| Allantoin (dog)                     |                                 | 1.1-3.0                           | 19         |

TABLE I (continued)

| 11122                                                                                                                                                                                    | E I (continued)                                                                                   |                                                                                                                             |                                                                                    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|
|                                                                                                                                                                                          | mg/I                                                                                              |                                                                                                                             |                                                                                    |
| Substance                                                                                                                                                                                | Average or representative value                                                                   | Range or<br>standard<br>deviation                                                                                           | References                                                                         |
| Carbohydrate and related substances                                                                                                                                                      |                                                                                                   |                                                                                                                             |                                                                                    |
| Glucose, fasting, venous blood Glucose, fasting, capillary blood Total reducing substances (as glucose) Lactic acid (resting) Pyruvic acid Citric acid a-Ketoglutaric acid Succinic acid | 83<br>93<br>1.0<br>2.5<br>0.8<br>0.5                                                              | S.D. 4<br>S.D. 3<br>90-120<br>8-17<br>0.77-1.23<br>1.9-2.8                                                                  | 28<br>28<br>29<br>30<br>31, 32<br>33, 34<br>35, 36<br>36, 37                       |
| Fat and related substances                                                                                                                                                               |                                                                                                   |                                                                                                                             |                                                                                    |
| Fat (total) Fatty acids (total), as stearic acid Phospho-Lipids (total) Lecithin                                                                                                         | 570<br>340<br>215                                                                                 | 360-820<br>200-800<br>123-293<br>50-200                                                                                     | 11<br>38<br>39, compar<br>38 and 40                                                |
| Cephalin<br>Sphingomyelin                                                                                                                                                                |                                                                                                   | 15-35                                                                                                                       | 39, compar<br>38 and 40<br>39, compar                                              |
| Lipid-P<br>Cholesterol, total<br>Cholesterol, free<br>Ketone bodies (as $\beta$ -hydroxybutyric acid)<br>Bile acids (as cholic acid)                                                     | 9.2<br>194<br>69                                                                                  | 6.1-14.5<br>107-320<br>26-106<br>0.33-0.87<br>0.2-3.0                                                                       | 38 and 40 11 11, 41 11, 41 42 43                                                   |
| Vitamins                                                                                                                                                                                 |                                                                                                   |                                                                                                                             |                                                                                    |
| Vitamin A Carotene (total carotenoids) Ascorbic acid Inositol Folic acid Biotin Pantothenic acid                                                                                         | 0.019-0.036<br>0.06-0.18<br>1.75·10 <sup>-3</sup><br>1.27·10 <sup>-3</sup><br>12·10 <sup>-3</sup> | 0.025<br>0.09<br>0.1-0.70<br>0.42-0.76<br>1.62-1.95·10 <sup>-3</sup><br>0.95-1.66·10 <sup>-3</sup><br>6-22·10 <sup>-3</sup> | 44<br>44<br>45<br>46<br>22<br>22<br>22                                             |
| Mineral constituents                                                                                                                                                                     |                                                                                                   |                                                                                                                             |                                                                                    |
| Na K Ca Mg Fe, men Fe, women Cu Mn (whole blood) Zn Cl                                                                                                                                   | 309<br>18<br>10<br>2.0<br>0.0945<br>0.0895<br>0.09                                                | 300-330<br>12-29<br>8.2-11.6<br>1.6-2.7<br>S.D. 0.0295<br>S.D. 0.0269<br>0.07-0.12<br>0.005-0.020<br>S.D. 0.16<br>350-375   | 47, 48, 49<br>47, 48<br>47<br>47<br>50<br>50<br>51<br>52<br>53, 54<br>47, see also |
| I (total) I (protein bound) F (whole blood) HCO <sub>3</sub> <sup>-</sup> (as vol. % CO <sub>2</sub> ) Phosphate, inorganic (as P) Phosphate, lipid (as P) SO <sub>4</sub> (as S)        | 0.007<br>61<br>3.7<br>9.2<br>1.57                                                                 | 0.006-0.008<br>0.006-0.008<br>0.04-0.15<br>55-75<br>2.9-4.3<br>6.1-14.5<br>1.00-1.85                                        | 55<br>56<br>57<br>29, 47, 49<br>47<br>11<br>58, 59, 60                             |

specific dynamic action, they are insignificant for the conditions of basal metabolism because the concentration of these substances in plasma is too low except during the period of absorption from the intestine.

The above list of 'relevant' substances can be simplified because lactate and pyruvate have very similar effects which are not additive, and only one of the two therefore needs to be added. Of the two, pyruvate has the advantage over L-lactate of being more readily available. Furthermore, all the acids of the tricarboxylic acid cycle have very similar effects<sup>65</sup>, as may be expected from their interconvertibility. Thus the addition of one of the acids should be sufficient. As for the choice, only three of the eight main acids of the cycle are readily available: citrate, succinate, and fumarate. Citrate has the disadvantage that it forms complexes with calcium and magnesium ions and thereby upsets the ionic balance of the medium. Succinate occupies a rather special position in that the first stage of its oxidation, the formation of fumarate, may proceed much more rapidly than the other stages of the cycle<sup>66</sup>; it may cause a brief period of rapid oxygen consumption followed by a steady rate at a lower level. There remains fumarate as the most suitable representative of the cycle.

From the point of view of tissue respiration the list of relevant metabolites can thus be reduced to four: glucose, pyruvate or lactate, fumarate, glutamate. As regards the concentrations to be used, experiments on kidney and brain cortex show that increasing the concentrations of pyruvate, lactate, fumarate or glutamate above 0.005 M makes no difference to the rate of respiration, except in very prolonged experiments. Glucose is usually not a limiting factor when its concentration is above 0.2%.

It is therefore suggested that serum be supplemented by adding isotonic substrate solutions in the following proportions:

100 parts of serum

- 3 parts of 0.16 M Na-pyruvate (or Na-L-lactate)
- 6 parts of o.1 M Na-fumarate
- 3 parts of 0.16 M Na-L-glutamate
- 5 parts of 0.3 M glucose

The mixture must be in equilibrium with a gas mixture containing about 5% CO<sub>2</sub>. The additions cause a dilution of the serum of about 15%. It is not possible when making additions to maintain both isotonicity and concentrations, and preference is given to the former.

The blood from which the serum is prepared should be cooled immediately after collection, otherwise the glycolytic activity of the blood cells will reduce the concentrations of glucose and bicarbonate and increase that of lactate. The bicarbonate content of the serum should be determined and if below 0.025 M it should be adjusted to that level by the addition of 1.3% NaHCO3 solution. It is advisable to sterilize the medium by passing it through a Seitz-filter.

Saline serum substitute (Medium I). Serum contains unknown and variable, and thus uncontrolled, constituents. It is furthermore difficult to obtain in sufficient quantities in the case of small animals, and heterologous serum may contain inhibitory antibodies. There is therefore a case for a serum substitute which can be easily prepared and whose composition is exactly known.

As a rule serum does not preserve the metabolic activities of isolated tissues more effectively than do saline media supplemented with substrates. The rates of the metabo-

References p. 267-269.

lic processes in isolated material which have so far been studied have usually been found to be of the same order in serum and in suitable saline media, at least for the usual experimental periods of under two hours. But some tissues, in particular brain, retina, choroid plexus, and foetal membranes, assume an opaque appearance on incubation in saline and tend to break up into fragments whilst appearance and texture remain unchanged in serum. The use of serum may therefore be advantageous in some investigations.

The earlier serum substitutes, such as RINGER's solution, were designed on an empirical basis. RINGER<sup>67</sup> tested the effect of various saline media on the beat of the isolated frog heart, and found that solutions containing certain quantities of Ca and K ions, in addition to NaCl maintained the beat for longer periods than NaCl solutions. Later, when precise data on the chemical composition of blood serum became available, saline media were modelled on these data<sup>68, 69, 70, 71</sup>. It has been found repeatedly that the closer the medium resembles serum the better does it maintain tissue activities in vitro. The previous attempts to copy the composition of serum, however, considered only the inorganic constituents and glucose.

The saline medium of Krebs and Henseleit<sup>71</sup> closely reproduces the inorganic constituents of mammalian serum except that the concentration of Cl is about 20% higher. A discrepancy of this kind is unavoidable in a purely inorganic medium because in serum a fraction of the anions, amounting to about 22 milliequivalents, consists of organic substances. Replacement of part of the NaCl by the Na salts of pyruvic (or L-lactic), fumaric and glutamic acids and addition of glucose eliminates the discrepancy in the chloride concentration and introduces the 'relevant' metabolites. The following composition is suggested for the saline serum substitute. Mix

```
1. 80 parts of 0.9% NaCl (0.154 M)
2. 4 parts of 1.15% KCl (0.154 M)
3. 3 parts of 0.11 M CaCl<sub>2</sub>
4. 1 part of 2.11% KH<sub>2</sub>PO<sub>4</sub> (0.154 M)
5. 1 part of 3.82% MgSO<sub>4</sub>.7 H<sub>2</sub>O
6. 21 parts of 1.3% NaHCO<sub>3</sub> (0.154 M); treated with CO<sub>2</sub> until p<sub>H</sub> is 7.4
7. 4 parts of 0.16 M Na-pyruvate (or L-lactate)
8. 7 parts of 0.1 M Na-fumarate
9. 4 parts of 0.16 M Na-L-glutamate
10. 5 parts of 0.3 M (5.4%) glucose

Prepared by neutralizing a solution of the acids with M NaHCO<sub>3</sub> solution
```

The mixture must be saturated with a gas mixture containing about 5% CO<sub>2</sub>. The stock solutions are approximately isotonic.

Solutions 7 to 10, unless sterilized, cannot be kept at room temperature. In the refrigerator they keep for about a week if gross bacterial infections are avoided.

Solutions I to 6 are mixed in the same proportion as the medium of KREBS AND HENSELEIT<sup>71</sup>, except that 80 parts NaCl solution instead of 100 parts are used. The difference of 20 ml is made up by the solutions 7 to 10. The concentrations of the constituents of this medium are shown in Table II. For comparison, data for human and rat sera are also given and it will be seen that the concentration of the electrolytes in the sera and the 'serum substitute' are very similar.

Sera of different mammalian species show relatively small variations except in the case of inorganic sulphate. Normal human serum is reported to contain I to I.5 mg SO<sub>4</sub> References p. 267-269.

| Substance                | Concentration in medium | Concentration in serum (milliequivalent/litre) |                                   |  |  |
|--------------------------|-------------------------|------------------------------------------------|-----------------------------------|--|--|
|                          | (milliequivalent/litre) | Human <sup>75</sup>                            | Rat <sup>76</sup> , <sup>77</sup> |  |  |
| Na                       | 141.0                   | 1.42                                           | 134                               |  |  |
| K                        | 5.93                    | 5                                              | 5.1                               |  |  |
| Ca                       | 5.08                    | 5                                              | 6.05                              |  |  |
| Mg                       | 2.36                    | 3                                              | 2.57                              |  |  |
| Cl                       | 104.8                   | 103                                            | 102                               |  |  |
| Phosphate* (inorganic)   | 2.22                    | 2                                              | 4.3                               |  |  |
| Sulphate (inorganic)     | 2.36                    | 1                                              |                                   |  |  |
| HCO <sub>3</sub>         | 24.9                    | 27                                             | 22                                |  |  |
| CO <sub>2</sub> (at 40°) | 1.0                     |                                                |                                   |  |  |
| Pyruvate                 | 4.9                     |                                                |                                   |  |  |
| Glutamate                | 4.9                     |                                                |                                   |  |  |
| Fumarate                 | 5.4                     |                                                |                                   |  |  |
| Total organic anions     | 20.7                    | 22                                             |                                   |  |  |
| Glucose                  | 9.2                     |                                                |                                   |  |  |

TABLE II

COMPARISON OF THE COMPOSITION OF SERUM AND SERUM SUBSTITUTE

(expressed as S) per 100 ml = 0.7 to 1.0 milliequivalent per litre<sup>58, 60</sup>; somewhat higher figures are given by  $Guillaumin^{72}$ . For dog, ox, goat, and horse figures between 3 and 4 mg S per 100 ml are reported<sup>73, 74</sup>. The serum substitute, being primarily intended for use with animal tissues, copies the sulphate concentration of animal serum. If a substitute for human serum is required half of the  $MgSO_4$  should be replaced by an equivalent amount of  $MgCl_2$  solution.

Owing to the danger of bacterial infection the solutions containing organic substances should be freshly prepared before use. A composite stock solution containing solutions 1–5 in the proportion stated and 3 parts of solution 6 is stable; the use of this mixture shortens the procedure for preparing the full medium.

Phosphate saline without Ca, and low in bicarbonate and  $CO_2$  (Medium II). Serum and the saline serum substitute may be inconvenient in the manometric measurement of respiration because they must be kept in equilibrium with gas mixtures containing about 5%  $CO_2$ . The measurement of the oxygen uptake is simpler and more accurate if the  $CO_2$  pressure of the gas phase can be kept near zero by absorbing the gas with alkali. A reduction of the  $CO_2$  pressure necessitates an equivalent reduction in the bicarbonate concentration if  $p_H$  is to remain within the physiological range. The following two types of media with low bicarbonate and  $CO_2$  concentrations have been in use:

Type A. The greater part of the bicarbonate-CO<sub>2</sub> buffer system is replaced by a phosphate buffer of the same p<sub>H</sub> and approximately equivalent concentration. As a high concentration of phosphate is incompatible with the physiological concentration of calcium ions the latter are usually omitted from such media. Ca-free phosphate salines are especially valuble as a medium for minced tissues and homogenates, as they give higher and steadier rates than calcium containing media<sup>78, 79, 5, 80, 81, 82</sup>.

Type B. The bicarbonate content is reduced to about one-tenth of the physiological value, with no change in the other constituents<sup>83, 84</sup>. Such a medium has the advantage of having a physiological concentration of calcium, but its buffering capacity is much below that of the media of Type A. The  $p_H$  is not precisely defined but indicator tests References p. 267–269.

<sup>\*</sup> In accordance with common usage one P is taken as 1.8 equivalent.

show that if the medium is shaken with respiring tissues which produce  $CO_2$  continuously  $p_H$  remains about 7.3. When the medium is allowed to stand for long periods or shaken without tissues  $p_H$  rises.

Comparative measurements have shown in many cases<sup>85, 86</sup> that tissues kept in these types of media respire at about the same rate as serum or saline serum substitutes containing Ca and bicarbonate in physiological concentrations.

A medium of the type A is prepared by omitting  $CaCl_2$  from medium I and replacing 18 parts of the NaHCO<sub>3</sub> solution by an isotonic phosphate buffer. Mix

```
83 parts of 0.9% NaCl
4 parts of 1.15% KCl
1 part of 2.11% KH<sub>2</sub>PO<sub>4</sub>
1 part of 3.82% MgSO<sub>4</sub>.7 H<sub>2</sub>O
3 parts of 1.3% NaHCO<sub>3</sub>
18 parts of Na-phosphate buffer (100 parts of 0.1 M Na<sub>2</sub>HPO<sub>4</sub> (1.78% Na<sub>2</sub>HPO<sub>4</sub>.2 H<sub>2</sub>O)
and 25 parts of 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (1.38% NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O))
4 parts of 0.16 M Na-pyruvate (or L-lactate)
7 parts of 0.1 M Na-fumarate
4 parts of 0.16 M Na-L-glutamate
5 parts of 0.3 M (5.4%) glucose
```

In this calcium-free medium the concentrations of Na, K, Mg, Cl and SO<sub>4</sub> approximate to those of serum; the concentration of phosphate is about 20 times higher, and that of HCO<sub>3</sub> about 10 times lower, than the physiological values.

Saline low in phosphate, bicarbonate, and  $CO_2$  (Medium III). Many previous observations indicate that calcium ions can influence the rate of respiration<sup>87, 88, 89, 90</sup>. It is therefore useful to have a medium which, like the synthetic serum substance, contains Ca in physiological concentrations but can, at the same time, be used in manometric experiments where  $CO_2$  is being absorbed by alkali. The medium suggested differs from medium II, apart from the inclusion of Ca, by a lower phosphate concentration and therefore lowering buffering capacity. These differences are necessitated by the limited solubility of Ca-phosphates. Mix

```
95 parts of 0.9% NaCl
4 parts of 1.15% KCl
3 parts of 0.11 M CaCl<sub>2</sub>
1 part of 2.11% KH<sub>2</sub>PO<sub>4</sub>
1 part of 3.82% MgSO<sub>4.7</sub> H<sub>2</sub>O
3 parts of 1.3% NaHCO<sub>3</sub>
3 parts of Na-phosphate buffer (as described for medium II)
4 parts of 0.16 M Na-pyruvate
7 parts of 0.1 M Na-fumarate
4 parts of 0.16 M Na-L-glutamate
5 parts of 0.3 M (5.4%) glucose
```

# $O_2$ pressure

In order to safeguard saturation of tissue slices with  $O_2$  it is generally necessary to have an  $O_2$  pressure of one atmosphere in the cup. It is known<sup>91, 92, 93</sup> that  $O_2$  of this pressure has a poisoning effect on some of the oxidative enzymes. As these effects are

small when the medium contains Mg ions and the period of observation is below 2 hours<sup>92</sup> they may be neglected in many cases.

# B. MEASUREMENT OF $Q_{\mathbf{O_2}}$ OF FIVE MAMMALIAN TISSUES

# 1. Procedure

At the start of this investigation it was decided to use medium II for the main measurements in preference to medium I because the absorption of  $CO_2$ , permissible in the case of medium II, simplifies the manometric technique. It was expected, on the basis of the results of previous investigators on similar media<sup>86, 85</sup>, that the three media would all give approximately the same  $Q_{O_2}$  values, but later comparative measurements of  $Q_{O_2}$  in the three different salines gave consistent differences in the case of some tissues, especially brain.

The measurements of the  $O_2$  uptake were carried out on sliced material in conical Warburg flasks of 20 to 26 ml capacity, provided with a centre well. The main compartment contained 4 ml medium, the centre well 0.3 ml 2 N NaOH, the gas space  $O_2$ . The temperature was  $40^\circ$ . All measurements were done in duplicate.

Five tissues, brain cortex, kidney cortex, liver, spleen, and lung, were examined. They were removed from the fasting animal as soon as possible after death and placed in ice-cold saline (medium III, in which the organic substrate solutions were replaced by an equal volume 0.9% NaCl). Slices were made free-hand or by the method of Deutsch<sup>91</sup>. During the slicing operation the tissue and razor blades were bathed in the modified medium III. Readings began after an equilibration period of 15 min and were continued at 5 or 10 min intervals for 45 min, so that the total period of incubation was 60 min.  $Q_{02}$  was calculated from the pressure change observed during the 45 min period of recording.

Abattoir material was collected in Dewar vessels containing 250 ml water, 250 g ice, 3.5 g NaCl, 15 ml 1.15% KCl and 12 ml o.11 M CaCl<sub>2</sub>. On addition of the tissue most of the ice melted and the resulting solution contained Na, K, Ca and Cl in approximately physiological concentrations. The material usually reached the laboratory within about one hour after killing. To test to what extent this treatment affected the rate of respiration samples of guinea pig and rat tissue were sliced immediately after death and another portion of the organ was subjected to storage in iced saline in the same way in the abattoir material, except that the period of storage was 4 hours. The results are shown in Table III. It will be seen that small losses of activity exceeding the limits of error occurred in storing guinea pig liver and guinea pig lung. As the delay in the examination of abattoir material was usually only one quarter of the time allowed for storing in this experiment it may be assumed that the losses in activity due to storage were negligible. If losses actually occurred the value given for abattoir material would be too low. Prolonged storage in iced saline caused considerable losses of activity. In an experiment in which guinea pig tissue was examined after a storage period of 24 hours  $Q_{02}$  of brain cortex fell 37%, of kidney cortex 11%, of liver 77%, of spleen 43%, and of lung 29%.

# 2. $Q_{\mathrm{O}_{\mathbf{2}}}$ in phosphate saline without calcium (medium II)

Data obtained on 9 different mammalian species are given in Table IV. Of each tissue 6 specimens were examined in the case of the rat, guinea pig, rabbit, sheep, cattle References p. 267–269.

and horse, 7 in the case of the mouse, 5 in the case of the dog and 2 in the case of the cat. The mean  $Q_{O_2}$  values for each tissue are given in Table V, together with mean values of heat production, for animals of the same average weight. The heat values are taken from Benedict<sup>95</sup>.

TABLE III  $\begin{tabular}{ll} EFFECT OF STORAGE OF TISSUES ON $Q_{O_2}$ \\ \end{tabular}$ 

|                           | $\mathbb{Q}_{\mathbf{O_2}}$ (average | Change in QC                  |                    |
|---------------------------|--------------------------------------|-------------------------------|--------------------|
| Tissue .                  | Sliced<br>immediately                | Stored 4 hours in iced saline | due to storing (%) |
| Brain cortex guinea pig   | — 25.I                               | 23.9                          | - 4·7              |
| Kidney cortex, guinea pig | — 32.9                               | 34.8                          | + 5·7              |
| Liver, guinea pig         | — 13.7                               | — 11.7                        | - 14.5             |
| Lung, guinea pig          | — 9.1                                | — 8.0                         | 12.1               |
| Liver, rat                | — 19.7                               | — 19.2                        | 2.5                |

 $\begin{array}{c} {\rm TABLE\ IV} \\ {\rm QO_2\ of\ 5\ Tissues\ of\ 9\ Mammalian\ species} \end{array}$ 

Slices suspended in medium II (phosphate buffered, no calcium); the data are the averages of duplicate determinations)

|    |                     |            |       |        | Age  | Weight          |                  | c      | $Q_{O_2}$   |              |              |
|----|---------------------|------------|-------|--------|------|-----------------|------------------|--------|-------------|--------------|--------------|
| No | o Species Breed Sex | Sex        | years | months | (1 ) | Brain<br>cortex | Kidney<br>cortex | Liver  | Spleen      | Lung         |              |
| I  | Mouse               | Albino     | m     |        |      | 0.035           | - 32.2           | _ 50.3 | - 22.2      | - 20.3       | -13.5        |
| 2  | ,,                  | ,,         | m     |        |      | 0.034           | - 30.2           |        | 20.2        |              | -10.6        |
| 3  | ,,                  | **         | m     |        |      | 0.012           | 30.0             |        | - 21.9      |              | - 10.4       |
| 4  | ,,                  | ,,         | m     |        |      | 0.028           | - 35.6           |        | - 23.7      |              | 11.6         |
| 5  | ,,                  | **         | m     |        |      | 0.015           | 30.2             |        | 20.9        | 16.3         | - 9.2        |
| 6  | ,,                  | ,,,        | m     |        |      | 0.009           | - 33.I           | 37.0   | - 25.6      | — I7.I       | 15.4         |
| 7  | "                   | **         | m     |        |      | 0.031           | - 39.6           | — 56.8 | - 27.5      | -19.0        | -13.6        |
| 1  | Rat                 | Albino     | m     |        |      | 0.22            | - 24.8           | - 33.8 | - 15.7      | 11.9         | -9.0         |
| 2  | ,,                  | **         | m     |        |      | 0.22            | - 25.6           |        | 13.9        |              | -9.6         |
| 3  | ,,                  | ,,         | m     | İ      |      | 0.20            | 21.5             | -41.6  | - 19.7      | - I 2.2      | 9.0          |
| 4  | ,,                  | ,,         | f     |        |      | 0.19            | -35.8            | -43.I  | - 16.5      | - 12.0       | 8.o          |
| 5  | ,,                  | .,         | f     |        |      | 0.24            | - 20.2           | -38.5  | - 14.7      | — I2.8       | 7.0          |
| 6  | ,,                  | **         | m     |        |      | 0.18            | 30.0             | -45.3  | - 22.6      | - 14.7       | 9.1          |
| I  | Guinea pig          |            | m     |        |      | 0.74            | - 28.7           |        | -13.6       | - 9.9        | -8.5         |
| 2  | ,,                  |            | f     |        |      | 0.54            | - 27.8           | - 31.3 | — I 3.7     | - 13.5       | <b>—</b> 7.6 |
| 3  | ,,                  |            | m     |        |      | 0.42            | 27.3             |        | 12.6        |              | - 9.2        |
| 4  | ,,,                 |            | m     |        |      | 0.40            |                  | - 31.0 |             |              | <b>−</b> 7.4 |
| 5  | .,,                 |            | m     |        |      | 0.49            | - 25.1           | - 32.9 | — I 3.5     | - 10.9       | -9.1         |
| 6  | ,,                  |            | m     |        |      | 0.41            | - 32.1           | - 34.3 | - 13.6      | 11.6         | - 9.4        |
| I  | Rabbit              | Chinchilla | f     |        |      | 1.03            | 26,6             | -31.4  |             | — I 5.5      | — 7·5        |
| 2  | ,,                  | **         | m     |        |      | 1.34            | 30.6             |        | — I 2.7     |              | - 7.9        |
| 3  | ,,                  | **         | m     |        |      | 1.31            | - 33.2           | - 36.7 | <b></b> 9.6 | - 19.1       | -9.1         |
| 4  | ,,                  | **         | f     |        |      | 1.23            | 27.0             | - 40.0 | 10.9        | - 12.5       | - 8.6        |
| 5  | ,,                  | .,,        | f     |        |      | 1.36            | 28.2             |        | 13.1        |              | - 8.2        |
| 6  | ,,                  | **         | f     |        |      | 1.11            | - 23.3           | - 34.8 | 11.8        | 9.3          | 6.5          |
| I  | Cat                 |            | m     |        |      | 3.11            | - 29.4           | - 21.0 | — I 3.3     | <b>—</b> 7⋅3 | — 3.I        |
| 2  | ,,                  |            | m     |        |      | 2.39            | - 24.3           | - 24.4 | - 13.0      | - 9.5        | -4.7         |

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TABLE IV (continued)

|    |         |                |     |       | Age    | Weight |                  |                  | $Q_{\mathbf{O_2}}$ |              |              |
|----|---------|----------------|-----|-------|--------|--------|------------------|------------------|--------------------|--------------|--------------|
| No | Species | Breed          | Sex | years | months | (kg)   | Brain<br>cortex  | Kidney<br>cortex | Liver              | Spleen       | Lung         |
| ı  | Dog     | Mongrel        | m   |       |        | 12.1   | - 20.7           | — 24.7           | — I 2.0            | 7.2          | 4.5          |
| 2  | ,,      | ,,             | m   |       |        | 12.5   | — 19.9           | 24.5             | - I 2.2            | 6.2          | - 4.9        |
| 3  | ,,      | ,,             | f   |       |        | 18.2   | 18.3             | 25.3             | — I 2.7            | 6.2          | - 3.9        |
| 4  | ,,      | ,.             | m   | }     |        | 22.5   | - 22.4           | - 32.0           | 10.5               | 6.5          | -4.6         |
| 5  | **      | ,,             | f   |       |        | 14.1   | - 24.5           | 28.7             | 11.1               | — 7.1        | 6.4          |
| ı  | Sheep   |                | f   | 2     | 6      | 72     | - 19.3           | - 26.9           | -9.3               | <b>—</b> 7.2 | 7.0          |
| 2  | ,,      |                | m   | 1     | 6      | 36     | - 18.6           | -31.3            | -8.3               | - 10.5       | 5.I          |
| 3  | ,,      | Cheviot        | f   | 4     | 0      | 63     | <del></del> 19.6 | - 27.I           | 7.8                | 6.8          | 4.7          |
| 4  | ,,      | Scotch         | m   | 1     | 8      | 36     | - 22.4           | <u> — 26.1 </u>  | - 9.2              | <b>−</b> 5.5 | <b>—</b> 5.2 |
| 5  | **      | Massam         | f   |       | 7      | 41     | - 20.2           | 29.9             | <del>-</del> 9.6   | <u> </u>     | <b>—</b> 5.8 |
| 6  | **      | Cheviot        | f   | I     | 6      | 45     | - 18.3           | - 23.6           | <del></del> 6.6    | -4.8         | <b>—</b> 4⋅7 |
| 1  | Cattle  | Cross          | f   | 3     | 6      | 320    | - 17.9           | - 22.8           | -8.2               | -4.2         | - 4.9        |
| 2  | ,,      | Short horn     | f   | 4     | 6      | 380    | - 20.1           | 22.0             | <del> 7.9</del>    | -4.2         | - 3.9        |
| 3  | ,,      | ,,             | f   | 4     | 6      | 510    | l — 16.5         | 23.6             | — 8.o              | -4.2         | <b>—</b> 3.9 |
| 4  | ,,      | 11             | m   | 4     | 0      | 440    | 1.81             | - 21.9           | <b>—</b> 7⋅3       | -4.9         | <b>—</b> 3⋅3 |
| 5  | ,,      | Aberdeen angus | m   | 2     | 0      | 570    | - 13.4           | 30.3             | <b>—</b> 9.6       | -4.4         | -4.8         |
| 6  | ,,      | Short horn     | f   | 3     | 0      | 320    | <b>— 17.3</b>    | - 19.2           | — 8.1              | -4.7         | - 4.2        |
| I  | Horse   | Shire          | f   | 25    | 0      | 610    | - 16.4           | - 18.2           | -6.1               | -4.4         | - 5.3        |
| 2  | ,,      | Cross          | f   | 15    | 0      | 610    | - 17.6           | - 21.0           | <b>—</b> 5.7       | -3.8         | 4.6          |
| 3  | ,,      | Shire          | m   | 10    | О      | 790    | - 17.4           | -23.5            | — 6.1              | -4.9         | - 4.1        |
| 4  | ,,      | ,,,            | m   | 6     | 0      | 790    | - 12.0           | - 22.6           | - 4.0              | -4.4         | 4.0          |
| 5  | ,,      | 1)             | f   | 7     | 0      | 760    | <u>— 16.5</u>    | 19.1             | <b>—</b> 5.9       | -4.1         | -4.6         |
| 6  | ,,      | ,,             | m   | 18    | 0      | 790    | - 14.1           | - 24.5           | -4.5               | <b>—</b> 3.8 | -4.1         |

TABLE V

average  $Q_{O_2}$  of 5 tissues of 9 mammalian species compared with average basal heat production ( $Q_{O_2}$  measured in medium II)

(The average  $Q_{02}$  values are computed from Table IV. The data on average basal heat production per kg bodyweight are taken from Benedict's graphs<sup>95</sup>. The heat data refer to animals of the average body weight given in the third vertical column).

| Species    | Bodyweigh   | Bodyweight (kg) |                 |                  | $Q_{O_2}$ |         |        |                                                  |  |  |
|------------|-------------|-----------------|-----------------|------------------|-----------|---------|--------|--------------------------------------------------|--|--|
|            | Range       | Mean            | Brain<br>cortex | Kidney<br>cortex | Liver     | Spleen  | Lung   | production/kg<br>bodyweight in<br>24 hours (Cal) |  |  |
|            |             |                 |                 |                  |           |         |        |                                                  |  |  |
| Mouse      | 0.012-0.035 | 0.021           | - 32.9          | 46.1             | — 23.I    | 16.9    | - 12.0 | 158                                              |  |  |
| Rat        | 0.18-0.22   | 0.21            | - 26.3          | 38.2             | - 17.2    | — I 2.7 | -8.6   | 100                                              |  |  |
| Guinea pig | 0.42-0.74   | 0.51            | - 27.3          | -31.8            | 13.0      | — II.6  | -8.5   | 82                                               |  |  |
| Rabbit     | 1.03-1.36   | 1.05            | - 28.2          | - 34.5           | 11.6      | - 14.2  | 8.0    | 60                                               |  |  |
| Cat        | 2.39-3.11   | 2.75            | - 26.9          | - 22.7           | 13.2      | -8.4    | -3.9   | 50                                               |  |  |
| Dog        | 12.1-22.5   | 15.9            | -21.2           | - 27.0           | 11.7      | 6.6     | -4.9   | 34                                               |  |  |
| Sheep      | 36-72       | 49              | - 19.7          | - 27.5           | -8.5      | 6.9     | 5.4    | 2.5                                              |  |  |
| Cattle     | 320-570     | 420             | - 17.2          | - 23.5           | -8.2      | -4.4    | 4.3    | 20                                               |  |  |
| Horse      | 610-790     | 725             |                 | - 21.5           |           |         | - 4.4  | 17                                               |  |  |
|            |             |                 | -               |                  |           |         |        |                                                  |  |  |

As already stated the values for  $Q_{02}$  were calculated from the pressure changes recorded between 15 and 60 min after the start of the incubation period. The rate of oxygen uptake often showed a progressive fall during the 45 min of observation, and

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 $Q_{02}$  values calculated for the period of incubation between 20 to 40 min were therefore as a rule somewhat higher than those given in the Table. In the case of brain and kidney the difference was no greater than 5%. In the case of the other three tissues it was of the order of 10%.

# 3. Qo2 in saline containing calcium and low in phosphate and bicarbonate (medium III)

On each of the 9 species I or 2 experiments were carried out in which  $Q_{O_2}$  was measured at the same time in media II and III. These experiments showed that in general the  $Q_{O_2}$  values calculated from the early readings (20 to 40 min) tended to be somewhat lower in medium III, but the progressive fall with time was less in this medium, and the  $Q_{O_2}$  values calculated for the I5 to 60 min period were within I0% the same in the case of kidney cortex, lung and spleen in all 9 species. On the other hand  $Q_{O_2}$  for brain, and some species of liver, was considerably lower in medium III, and of these 2 tissues further specimens were examined. The results are given in Tables VI and VII.

TABLE VI  $_{\mathrm{QO_2}}$  of brain cortex and liver of 9 mammalian species (Slices suspended in medium III (low in bicarbonate; containing calcium); the data are the averages of duplicate determinations).

|    | Species    |            |     | A     | ge                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Weight (kg) | Q               | 202           |
|----|------------|------------|-----|-------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|-----------------|---------------|
| No |            | Breed      | Sex | years | months                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |             | Brain<br>cortex | Liver         |
| I  | Mouse      | Albino     | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.045       | 19.9            | - 15.6        |
| 2  | ,,         | ,,         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.044       | - 22.9          |               |
| 3  | ,,         | 91         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.031       | - 24.4          |               |
| 4  | ,,,        | ,,         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.040       | 23.2            | 20.2          |
| 5  | ,,         | 1)         | f   |       | 1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | 0.009       | 24.3            | - 22.3        |
| 6  | ,,         | Black      | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.020       |                 | 18.3          |
| 7  | ,,         | ,,         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.013       |                 | 20.3          |
| I  | Rat        | Albino     | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.27        | - 20.6          | 12.3          |
| 2  | ,,         | ,,,        | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.18        | 20.8            | — 17.4        |
| 3  | ,,         | 11         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.37        | 17.9            | - 13.9        |
| 4  | ,,         | 11         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.24        | 18.3            | - 14.0        |
| 5  | ,,         | **         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.25        | - 18.5          | 15.5          |
| I  | Guinea pig |            | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.58        | 18.5            | 6.07          |
| 2  | ,,         |            | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.58        | 20.0            | <b>—</b> 6.60 |
| 3  | ,,,        |            | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.41        | - 17.4          | - 9.50        |
| 4  | ,,         |            | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.28        | 16.4            | 11.6          |
| 5  | ,,         |            | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.50        | 15.8            | <b>—</b> 9.95 |
| I  | Rabbit     | Chinchilla | f   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 1.11        | - 15.3          | 7.6           |
| 2  | ,,         | 11         | f   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 0.93        | 15.0            |               |
| 3  | ,,         | ,,         | f   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 1.12        | — 15.6          |               |
| 4  | ,,         | **         | f   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 1.53        | — <b>15.</b> 6  |               |
| 5  | ,,         | + 3        | f   |       | The state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the state of the s | 1.35        | - 14.2          | 6.9           |
| I  | Cat        |            | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 3.11        | - 14.9          | -9.4          |
| 2  | "          |            | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 2.39        | 16.1            | 11.0          |
| I  | Dog        | Mongrel    | f   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 18.2        | — 16.o          | 12.9          |
| 2  | ,,         | ,,         | m   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 22.5        | 13.8            | -9.5          |
| 3  | ,,         | 7.1        | f   |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 14.1        | - 14.5          | - 9.9         |
| _  |            |            |     |       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |             |                 |               |

| TABLE VI (continu | (bar |
|-------------------|------|

|     | Species |                        |     | . A   | Age    | Weight (kg) | Q               | $\mathcal{O}_2$ |
|-----|---------|------------------------|-----|-------|--------|-------------|-----------------|-----------------|
| No. |         | Breed                  | Sex | years | months |             | Brain<br>cortex | Liver           |
| 1   | Sheep   | Scotch                 | m   | 0     | 9      | 27          | -12.4           | — 7·2           |
| 2   | ,,      | ,,,                    | f   | 2     | 6      | 36          | 10.0            | -8.€            |
| 3   | ,,      | Cheviot                | f   | I     | 6      | 45          | 10.0            | 6.7             |
| 4   | **      | Sussex                 | f   | О     | 8      | 41          | - IO.I          | 3.              |
| 5   | **      | Lincolnshire crossbred | m   | 0     | 8      | 27          | 14.1            | 5               |
| 1   | Cattle  | Shorthorn              | m   | 4     | 6      | 280         |                 | 2.6             |
| 2   | ,,      | Shorthorn crossbred    | m   | 3     | 0      | 290         | - 12.3          | 4.              |
| 3   |         | Shorthorn              | f   | 3     | 0      | 320         | - 15.4          | <b>—</b> 3.     |
| 4   | .,      |                        | m   | 2     | 6      | 38o         | 10.8            | -4.0            |
| 5   | ,,      | ,,,                    | f   | 4     | - 6    | 320         | - 10.6          | - 2.            |
| 6   | **      | ",                     | f   | 3     | 6      | 290         | -11.4           | 3.              |
| 1   | Horse   | Shire                  | m   | 18    | 0      | 790         | 10.0            | 1.5             |
| 2   | ,,      | ,,                     | f   | 7     | О      | 760         | 8.64            | - 2.            |
| 3   | ,,      | Belgian                | m   | 15    | 0      | 710         | - 13.7          | 2.              |
| 4   | 1.      | Shire                  | f   | 10    | 0      | 760         | - 11.5          | 3.              |
| 5   | ,,      | ,,                     | m   | 13    | 0      | 760         | 8.78            | 2.              |

TABLE VII

AVERAGE  $Q_{O_2}$  OF BRAIN CORTEX AND LIVER OF 9 MAMMALIAN SPECIES COMPARED WITH AVERAGE BASAL HEAT PRODUCTION ( $Q_{O_2}$  MEASURED IN MEDIUM III)

(The average  $Q_{02}$  values are computed from Table VI. The average basal heat production is taken from Benedict's  $^{95}$  graphs

| Species    | Body w<br>(kg |       | Qo           | Basal heat pro-<br>duction/kg body- |                   |  |
|------------|---------------|-------|--------------|-------------------------------------|-------------------|--|
|            | Range         | Mean  | Brain cortex | Liver                               | weight in 24 hour |  |
| Mouse      | 0.009-0.045   | 0.038 | 22.9         |                                     | 145               |  |
|            | 0.009-0.045   | 0.026 |              | <del> 1</del> 9.3                   | 152               |  |
| Rat        | 0.176-0.365   | 0.26  | - 19.2       | -14.6                               | 92                |  |
| Guinea pig | 0.279-0.58    | 0.44  | - 17.4       | -9.5                                | 85                |  |
| Rabbit     | 0.93-1.53     | I.2I  | 15.1         | 7.6                                 | 57                |  |
| Cat        | 2.39-3.11     | 2.75  | — I 5.5      | 10.2                                | 50                |  |
| Dog        | 14.1-22.5     | 18.3  | 14.8         | IO.8                                | 31                |  |
| Sheep      | 27-45         | 35    | 11.3         | -6.2                                | 27                |  |
| Cattle     | 280-380       | 320   | I 2. I       | 3.6                                 | 21                |  |
| Horse      | 710-790       | 760   | 10.5         | 2.6                                 | 17                |  |

# 4. $Q_{O_2}$ in saline serum substitute (medium I)

In order to decide whether the difference between the  $Q_{\rm O_2}$  values obtained for brain and liver in media II and III were due to the differences in the calcium content, or in the bicarbonate and phosphate content, comparative measurements were made on the same tissue sample in media I, II and III. The 'indirect' method of Warburg<sup>96</sup> was used for the measurements in medium I, in preference to those of Dickens and Simer<sup>97</sup> or Dixon and Keilin<sup>98</sup>, because with this method it is possible to follow the time course of the oxygen uptake. Duplicate sets of vessels were used in each measurement.  $Q_{\rm O_2}$  was again calculated for the 15 to 60 min period of incubation. The results of the comparative measurements are given in Table VIII.

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 ${\rm TABLE\ VIII}$  comparative measurements of  $\varrho_{\rm O_2}$  in 3 different saline media

|                          |            | $Qo_2$                                                                                        |                                                     |                                                           |  |  |  |
|--------------------------|------------|-----------------------------------------------------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------------|--|--|--|
| Tissue                   | Species    | Medium I (Containing physiological concentrations of HCO <sub>3</sub> ′ and CO <sub>2</sub> ) | Medium II<br>(Phosphate<br>buffered,<br>no calcium) | Medium III<br>(Low in bicarbonate;<br>containing calcium) |  |  |  |
| Brain cortex " " " " " " | Guinea pig | — 18.6                                                                                        | - 34.2                                              | — 16.4                                                    |  |  |  |
|                          | Rabbit     | — 17.5                                                                                        | - 23.9                                              | — 15.6                                                    |  |  |  |
|                          | Sheep      | — 13.5                                                                                        | - 17.6                                              | — 12.4                                                    |  |  |  |
|                          | Cattle     | — 9.9                                                                                         | - 15.9                                              | — 10.8                                                    |  |  |  |
|                          | Horse      | — 13.7                                                                                        | - 16.5                                              | — 13.7                                                    |  |  |  |
| Liver " " " "            | Mouse      | — 19.6                                                                                        | 18.6                                                | 20.2                                                      |  |  |  |
|                          | Guinea pig | — 10.8                                                                                        | 12.2                                                | 11.6                                                      |  |  |  |
|                          | Rabbit     | — 10.3                                                                                        | 9.9                                                 | 8.1                                                       |  |  |  |
|                          | Sheep      | — 5.2                                                                                         | 6.0                                                 | 5.1                                                       |  |  |  |
|                          | Cattle     | — 3.6                                                                                         | 4.7                                                 | 3.5                                                       |  |  |  |
|                          | Horse      | — 2.7                                                                                         | 3.2                                                 | 2.9                                                       |  |  |  |

### C. DISCUSSION OF RESULTS

# 1. Comparison of the $Q_{\mathrm{O}_2}$ values obtained in the 3 media

Kidney cortex, spleen and liver gave about the same  $Q_{02}$  in all three media, but differences exceeding 10% were found in brain cortex and in liver. A comparison of the data from Tables V and VII (see Table IX) shows that the average  $Q_{02}$  values for brain cortex in medium II were between 37 and 87% higher than those obtained in medium III. In the case of the liver the differences were smaller; they are of doubtful significance in the small animals (mouse, rat) and increase approximately (though not strictly) parallel with the body weight of the species, being greatest in cattle and horse.

TABLE IX DIFFERENCES IN THE AVERAGE  $Q_{O_2}$  values in media ii (containing no ca) and iii (containing ca) (The figures show the level of  $Q_{O_2}^{Medium\ II}$  expressed as per cent of  $Q_{O_2}^{Medium\ III}$ , calculated from the data in Tables V and VII).

| Species    | $\frac{Q_{O_2}^{\text{Medium III}}}{Q_{O_2}^{\text{Medium III}}} \cdot 100$ |       |  |  |  |
|------------|-----------------------------------------------------------------------------|-------|--|--|--|
|            | Brain cortex                                                                | Liver |  |  |  |
| Mouse      | 144                                                                         | 120   |  |  |  |
| Rat        | 137                                                                         | 118   |  |  |  |
| Guinea pig | 157                                                                         | 137   |  |  |  |
| Rabbit     | 187                                                                         | 153   |  |  |  |
| Cat        | 174                                                                         | 129   |  |  |  |
| Dog        | 143                                                                         | 108   |  |  |  |
| Sheep      | 174                                                                         | 137   |  |  |  |
| Cattle     | 142                                                                         | 227   |  |  |  |
| Horse      | 150                                                                         | 208   |  |  |  |

According to Table VIII, media I and III give approximately the same  $Q_{O_2}$  values. The considerable differences in the concentration of bicarbonate,  $CO_2$  and phosphate in these two media have thus no major effect on the  $Q_{O_2}$  under the conditions tested. Since medium I resembles the physiological environment more closely than the other media,  $Q_{O_2}$  values obtained with this medium might be regarded as approximating more closely to the physiological value than higher values found for brain, and the liver of the larger animals in medium II. The latter are not likely to be standard  $Q_{O_2}$  values but no definite statement can be made on this point because reliable data on the  $O_2$  consumption of tissue  $in\ vivo$  are too scanty. In experiments of Noell and Schneider<sup>99</sup> the  $O_2$  consumption of dog brain cortex  $in\ vivo$  was 4.5 ml per minute per 100 g fresh weight, and on the assumption that the dry weight of dog brain cortex is 21% of the fresh weight<sup>100</sup>  $Q_{O_2}$   $in\ vivo$  was -12.9. This value is in good agreement with the figure of -14.8 found in medium III (Table VII) and favours the view that the values found for brain in the Ca-free medium II are abnormally high.

Effects of calcium in the  $Q_{O_2}$  of slices and homogenates have been described before and have recently been reviewed by CUTTING AND McCance<sup>90</sup>. Elliott and Libet<sup>6</sup> found that Ca depresses the initial rate of respiration of brain homogenates, but delays the falling off at the later stages of incubation, thus steadying the rate of respiration. It does not seem to have been noted before that the effect of Ca on tissue slices is greater in brain than in other tissues.

Whilst there is some uncertainty as to which of the values obtained in the different media constitute the 'basal'  $Q_{02}$ , it should be stated that the conclusions drawn in the following sections are not affected by this uncertainty.

# 2. Absolute level of Qo2

The  $Q_{02}$  values in all 3 media tend to be considerably higher than the values reported in the literature for saline media<sup>101</sup>, especially in the case of brain, liver and kidney. However, no strict comparison is possible because different substrates were used in previous measurements. The combination of substrates added in the present experiments give, in general, higher values than the substrates added in most previous work (glucose or lactate). The  $Q_{02}$  values observed in the new media are of the same order as the highest values recorded for serum. Thus the intention to include in the new media the substances in serum which stimulate respiration<sup>85</sup> seems to have been accomplished.

# 3. $Q_{\rm O_2}$ and body size

General survey. The data given in Tables IV, V and VII show that the  $Q_{02}$  values of the tissues of the larger species are, in general, somewhat lower than the homologous values of the smaller species. But there are many exceptions to this general rule. No strict parallelism exists between the  $Q_{02}$  values of the homologous tissues and the basal heat production per unit body weight of the intact animal. The  $Q_{02}$  values for brain, kidney, spleen, and lung change much less, and those for liver slightly less, with the body weight than the rate of basal heat production. Neither is there a simple correlation between body size and  $Q_{02}$  within the same species. The body weights of the 7 mice listed in Table IV varied between 9 and 35 g and that of the 5 mice listed in Table VI between 9 and 45 g. There were variations between 36 and 72 kg in the body weight of the 6 sheep of the first series. These differences of the body weight within one species are not reflected by differences in the  $Q_{02}$  values, with the doubtful exception of the

values for brain in Table VI, where the brains of the 2 smaller sheep show higher values than the 3 brains from the larger animals.

Brain cortex. In the largest species (horse) the average  $Q_{02}$  of brain cortex was about half the average  $Q_{02}$  value of the smallest species (mouse) namely 48% for the measurements in medium II, and 46% for the measurements in medium III. In contrast, the basal heat production per kg bodyweight of the horse is only 11% and 12% respectively of that of the mouse.

Kidney cortex. The changes of the  $Q_{O_2}$  values from species to species in this tissue were similar to those of brain cortex. The average  $Q_{O_2}$  value of horse kidney cortex was 47% of that of mouse kidney. The average  $Q_{O_2}$  value for sheep kidney was only 14% below that for guinea pig kidney, whilst the basal heat production per kg. bodyweight of the sheep is only 37% of the guinea pig. Thus the decrease of the  $Q_{O_2}$  values with body size was again much smaller than the decrease in the rate of the basal heat production.

Spleen, lung. For the horse the  $Q_{O_2}$  value of spleen tissue was about a quarter, and for lung about one third, of the corresponding values for the mouse. In these two tissues the discrepancies between the changes in  $Q_{O_2}$  and the changes in basal heat production in relation to body size are thus not as great as in brain and kidney, but they are still considerable.

Liver. Liver shows greater  $Q_{02}$  changes with body weight than any other tissue tested, especially in medium III (Tables V and VII). In medium II  $Q_{02}$  of horse liver was 23%, and in medium III it was 13.5% of that of mouse liver. Thus, when comparing the  $Q_{02}$  values obtained in medium II for these two species, about the same percentage change is found as for the basal rate of heat production. But the parallelism over the whole series of species is very imperfect. For example, the  $Q_{02}$  values for guinea pig, cat and dog are about the same (-9.5; -10.2; -10.8), whilst the basal rate of heat production shows a progressive fall with body weight (85; 50; 31).

The changes of  $Q_{O_2}$  of liver with body weight reported in this paper are similar to those found by Kleiber<sup>3</sup>, but owing to the differences in the media used the present  $Q_{O_2}$  values are all higher than those reported by Kleiber.

# 4. Rôle of muscle tissue in chemical temperature control

As the rate of respiration of a number of homologous tissues of animals of different sizes fails to show a strict parallelism with the basal rate of heat production of the intact body, it remains to be explained how the characteristic differences in the basal rates of heat production of animals of different sizes arise. One kind of explanation is contained in various publications by Kestner<sup>102</sup>, <sup>103</sup> and Blank<sup>104</sup>, who stated that the proportion of highly active organs is somewhat greater in the body of small animals than in that of large animals. He expressed the view that the "relative size of the brain and the large glands can give a complete explanation of the different heights of metabolism in different animals<sup>103</sup>". This view is not substantiated by quantitative measurements and such data as are available cannot be reconciled with Kestner's hypothesis (see Kleiber<sup>4</sup>).

An alternative explanation is offered by the conception that the relation between  $Q_{02}$  and body weight found for the 5 tissues tested does not hold for every tissue; that there is at least one major tissue whose "basal"  $Q_{02}$  changes with the body weight approximately parallel with the basal heat production; that this organ is the striated musculature.

The substance of this conception is, of course, not new in that it is commonly References p. 267-269.

accepted that the muscles play a leading part in the regulation of heat production. Evidence in support of this conception is the increased muscular activity on exposure to cold, manifesting itself by increased tension and shivering, and the failure of the curarized animal to maintain the physiological temperature level on exposure to cold. It has not been directly demonstrated that the basal respiration of the musculature varies with body size in the postulated fashion, and no satisfactory experimental procedure has as yet been devised to carry out the necessary measurements. Data obtained on isolated muscles bear no simple relation to the basal respiratory rate of the muscle in situ because the  $Q_{02}$  of muscle depends more than that of any other tissue on the state of activity of the tissue. Activity may cause a thirty-fold rise of the resting rate of respiration (Barcroft<sup>105</sup>, Meyerhof<sup>106</sup>). As the state of activity is controlled by the higher nervous centres detachment from the nervous system is bound to affect the rate of respiration.

# 5. Factors determining the level of tissue respiration

If body size is not a major factor determining the  $Q_{02}$  of the 5 tissues tested it remains to be examined which other factors control the level of respiration of these tissues. As the respiration of living tissues primarily serves to supply energy, the level of tissue respiration is expected to be determined by the energy requirements. A variety of factors contribute to the requirements. They may be classed in three groups:

- 1. Energy is required when tissues perform mechanical, osmotic, chemical, or other kinds of external work.
- 2. Energy is required to maintain structures which are thermodynamically unstable. An example is the maintenance of concentration gradients between tissue and blood plasma of readily diffusable substances, such as inorganic ions, amino acids, coenzymes.
  - 3. Energy is required to maintain the body temperature.

Energy generated for the first two purposes always yields heat as a by-product and in homeotherms this heat is partly, or wholly, utilised to maintain the body temperature. In an organism performing some physical exercise, and living at a temperature not far removed from that of the body temperature, the heat arising as a by-product may be enough for the upkeep of the body temperature. In a cold environment the heat arising as a by-product in a resting organism may no longer be sufficient to maintain the body temperature, and extra heat has to be formed. It is reasonable to assume that the highly differentiated cells whose task it is to carry out specialised functions, as do those of brain or the glands, are designed to deal solely with these specialized functions rather than to act as heat generators in the case of exceptional loss of heat. The extra source of heat might be expected to be the muscle tissue which, for other reasons, has the capacity of varying the rate of heat production. If this assumption is correct, in other words, if the level of respiration of highly specialized tissues is determined by the energy requirement falling under categories (1) and (2), it is to be expected that the rate of energy production of the highly differentiated cells is not dependent on the size of the animal, because the energy needed for the performance of a given piece of work is independent of the size of the body.

However, somewhat different from the question of energy requirements of the highly differentiated *cells* is the problem of the energy requirements of organs as a whole. Homologous organs of different species have by no means identical structures. For *References p. 267–269*.

example, in a larger species, tissue structures accessory to the main functional cells are bound to constitute a relatively larger portion of the organ than in the homologous tissue of a smaller animal. Such accessory structures are, among others, blood vessels, glandular ducts, connective tissues.

Thus some changes of the  $Q_{O_2}$  values with body size may be expected in homologous tissues even if the  $Q_{O_2}$  of homologous cells is the same. In general the change will be a decrease with body size because cells with lower respiration, like those of connective tissue, blood vessels and ducts, are bound to became more preponderant in the larger species. The changes in the  $Q_{O_2}$  with body size, seen in Tables V and VII, may in part be due to this factor.

### SUMMARY

The factors affecting the rate of respiration in isolated tissues are discussed with reference to the measurement of a "standard rate" of metabolic processes *in vitro*. Media for the suspension of tissues are devised; their composition is essentially based on the available analytical data for blood plasma.

 $Q_{O_2}$  of liver, brain cortex, kidney cortex, spleen, and lung was measured for 9 mammalian species of different body size (mouse, rat, guinea-pig, rabbit, cat, dog, sheep, cattle, horse). Three different media were used ("phosphate saline without Ca", "saline low in phosphate, bicarbonate and  $CO_2$ " and "saline serum substitute" containing physiological concentrations of inorganic ions in addition to organic substrates). Kidney cortex, spleen, and liver gave about the same  $Q_{O_2}$  values in all three media.  $Q_{O_2}$  for brain cortex was for all species higher in the medium containing no Ca, the average level being 37–87% higher.  $Q_{O_2}$  for liver was also higher in the absence of Ca, especially in the larger species.

 $Q_{O_2}$  values of the tissues of larger animals were in general somewhat lower than the homologous values of the smaller species but no strict parallelism between  $Q_{O_2}$  values and basal heat production of the intact animal was found. The  $Q_{O_2}$  values for most tissues changed much less with the body weight than the rate of basal heat production.

The absolute level of  $Q_{O_2}$  in the new media (which apart from glucose contain pyruvate, fumarate and L-glutamate) was higher than the values reported in the literature for saline media. They are of the same order as the highest values recorded for serum.

The characteristic differences in the basal rate of heat production in animals of different size are to be attributed mainly to variation in the  $Q_{02}$  of the musculature. It is suggested that the  $Q_{02}$  of tissues other than muscle is in the first place governed by the specific energy requirements of the tissues, and not by the heat requirements of the whole body.

### RÉSUMÉ

Les facteurs qui influencent la vitesse de la respiration dans les tissus isolés sont discutés par rapport aux mesures d'une "vitesse standard" des processus métaboliques in vitro. L'auteur décrit des milieux de suspension de tissus; leur composition se base essentiellement sur les données analytiques connucs pour le plasma sanguin.

Le  $Q_{O_2}$  a été déterminé pour le foie, le cortex du cerveau et du rhein, la rate et le poumon de 9 espèces de mammifères de taille différente (souris, rat, cobaye, lapin, chat, chien, mouton, bétail, cheval). Trois milieux différents ont été employés, le "phosphate salin sans Ca", "la solution saline faible en phosphate, bicarbonate et  $CO_2$  et "la solution saline, remplaçant le sérum" qui contient des concentration physiologiques d'ions inorganiques en plus du substrat organique. Dans les trois milieux le cortex rhénal, la rate et le foie donnèrent environ les mêmes valeurs de  $Q_{O_2}$ . Pour le cortex cervical ce facteur était plus élevé pour toutes les espèces animales examinées dans les milieux exempts de Ca. En moyenne les valeurs étaient de 37 à 87% supérieures. Pour le foie le  $Q_{O_2}$  était aussi supérieur en absence de Ca, surtout dans les espèces plus grandes.

En général les valeurs de  $Q_{O_2}$  étaient plus basses pour les tissus des animaux plus grands que les valeurs homologues pour les animaux plus petits. Cependant nous n'avons pas trouvé un parallè-

lisme stricte entre les valeurs de  $Q_{02}$  et la production de chaleur des animaux intacts.

Dans les nouveaux milieux (qui, à part le glucose, contiennent du pyruvate, du fumarate et du L-glutamate) le niveau absolu du  $Q_{02}$  était plus élevé que les valeurs rapportées dans la littérature pour une solution saline. Elles sont du même ordre que les valeurs les plus élevées rapportées dans la littérature pour le sérum.

Les différences caractéristiques dans la vitesse de base de la production de chaleur des animaux de differente taille doivent être attributées surtout à la variation du  $Q_{O_2}$  de la musculature. L'auteur suggère l'idée que le  $Q_{O_2}$  des tissus autres que le muscle serait gouverné en premier lieu par les besoins spécifiques d'énergie des tissus et non par les besoins de chaleur du corps entier.

### ZUSAMMENFASSUNG

Die Faktoren, welche die Geschwindigkeit der Atmung in isolierten Geweben beeinflussen, werden diskutiert und zwar mit Rücksicht auf die Messungen einer "Standardgeschwindigkeit" metabolischer Prozesse *in vitro*. Medien für Gewebesuspensionen werden vorgeschlagen, deren Zusammensetzung sich hauptsächlich auf die für Blutplasma bekannten analytischen Werte gründet.

Der Faktor QO2 von Leber, Gehirnrinde, Nierenrinde, Milz, und Lunge wurde für 9 Säugetierarten verschiedener Körpergrosse (Maus, Ratte, Meerschweinchen, Kaninchen, Katze, Hund, Schaf, Vieh, Pferd) bestimmt. Drei verschiedene Medien wurden verwendet, nämlich "Phosphat-Salz-Lösung ohne Ca", "Salzlösung mit geringem Gehalt an Phosphat, Bicarbonat und CO2" und "Salzlösung-Serumersatz", welche ausser anorganischen Ionen in physiologischen Konzentrationen organische Substrate enthält. Nierenrinde, Milz und Leber gaben ungefähr dieselben QO2-Werte in allen drei Medien. Der QO2 der Gehirnrinde war für alle Arten in dem Ca-freien Medium höher und zwar betrug der Unterschied durchschnittlich 37 bis 87%. Auch für die Leber lagen die Werte höher in Abwesenheit von Ca und zwar insbesondere in den grösseren Arten.

Im Allgemeinen lagen die  $Q_{O_2}$ -Werte der Gewebe grösserer Tiere etwas niedriger als die homologen Werte kleinerer Arten; es konnte aber kein strenger Parallelismus zwischen den  $Q_{O_2}$ -Werten und der Wärmebildung unverletzter Tiere gefunden werden. Die  $Q_{O_2}$ -Werte der meisten Gewebe varieren viel weniger mit dem Körpergewicht als die Geschwindigkeit der Wärmebildung.

Die absolute Lage der  ${\rm O}_{\rm O2}$ -Werte war in den neuen Medien, die ausser Glucose noch Pyruvat, Fumarat und L-Glutamat enthalten, höher als die in der Literatur für Salzlösungen beschriebenen Werte. Sie sind von der gleichen Grössenordnung wie die höchsten in der Literatur für Serum angeführten Werte.

Die charakteristischen Unterschiede in der Geschwindigkeit der Wärmebildung von Tieren verschiedener Körpergrösse müssen hauptsächlich auf die Änderungen des  $Q_{\rm O_2}$  in der Muskulatur zurückgeführt werden. Die Ansicht wird ausgesprochen, dass der  $Q_{\rm O_2}$  von anderen Geweben als Muskeln an erster Stelle durch die Energiebedürfnisse der Gewebe und nicht durch den Wärmebedarf des ganzen Körpers bedingt wird.

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# SYNTHÈSE ET UTILISATION DE L'AMIDON CHEZ UN FLAGELLÉ SANS CHLOROPHYLLE INCAPABLE D'UTILISER LES SUCRES

par

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On connaît un certain nombre de microorganismes incapables d'utiliser les sucres comme aliment carboné. Ce sont des bactéries comme les Moraxella ou des flagellés appartenant à des groupes divers et possédant ou non de la chlorophylle: Euglena et Astasia (Eugléniens), Polytoma, Chlorogonium et Hyalogonium (Chlamydomonadines). Les flagellés synthétisent tous des réserves glucidiques figurées: paramylon chez les Eugléniens, amidon chez les autres, dont ils sont très abondamment pourvus à certains stades de l'évolution des cultures et qui diminuent ou même disparaissent à d'autres.

Le problème de la synthèse et de l'utilisation des polysaccharides par un microorganisme incapable d'utiliser les sucres se trouvait posé. Nous avons étudié de ce point de vue le flagellé Polytomella coeca. Nos résultats ont été exposés dans une note préliminaire¹. Polytomella est un flagellé sans chlorophylle. Il se développe en culture bactériologiquement pure dans des milieux synthétiques avec un sel d'ammonium comme aliment azoté, de l'acide acétique ou de l'éthanol comme aliment carboné énergétique, les sels minéraux habituels et deux facteurs de croissance, le méthyl-4  $\beta$ -hydroxyéthyl-5 thiazole et la méthyl-2 amino-4 aminométhyl-5 pyrimidine². Il possède la propriété de se multiplier bien entre  $p_H$  3.0 et 8.0³, propriété précieuse qui a déjà été mise à profit pour l'examen d'un certain nombre de problèmes⁴.

Aucun sucre ne peut remplacer l'acide acétique ou l'éthanol pour la nutrition carbonée. En particulier, ni le glucose, ni le maltose, ni le saccharose, ni le tréhalose ne sont utilisables. Des essais pour obtenir des mutants utilisant les sucres ont échoué. Dans des milieux pauvres en aliment carboné, par exemple, éthanol à 1 p. 5000 ou à 1 p. 10000, c'est la teneur en éthanol qui limite la croissance. Dans ces milieux pauvres, les flagellés restent vivants pendant plus de 3 mois. Si un mutant capable d'utiliser un glucide apparaissait dans un milieu pauvre additionné de glucose, il y aurait multiplication abondante. Nous n'avons jamais observé ce phénomène. Il n'est naturellement pas possible d'exclure son existence, mais l'on peut dire que la probabilité de l'apparition d'un mutant utilisant les glucides est faible. Enfin, du glucose ajouté à des cultures en voie de développement en présence d'éthanol ne disparaît pas.

L'incapacité d'utiliser les glucides pour la nutrition est donc absolue. Comment les flagellés synthétisent-ils l'amidon, et comment l'utilisent-ils s'ils sont incapables de métaboliser les glucides. Tel est le problème qui va être examiné.

## TECHNIQUE

Le milieu suivant a été utilisé: sulfate d'ammonium r g,  $SO_4Mg + 7H_2O$  o.1 g,  $PO_4H_2K$  o.5 g, acétate de sodium r g, éthanol 3 ml, thiamine o.or mg, eau bidistillée r l, soude pour  $p_H$  7.o. Après stérilisation on ajoute du fer sous forme de citrate ferrique, stérilisé à part, pour obtenir une concentration finale de 10 mg/l.

On utilise pour l'alimentation carbonée un mélange d'acétate de sodium et d'éthanol afin que le  $p_H$  ne s'éloigne pas trop de la neutralité. Le  $p_H$  augmente en effet notablement lorsque l'aliment carboné est représenté par de l'acétate de sodium — libération d'ions  $Na^-$  — et diminue lorsque l'aliment carboné est représenté par de l'éthanol par suite de la libération non compensée des ions  $SO_4^-$  de l'aliment azoté. Avec le mélange utilisé, il n'y a pas de variation importante du  $p_H$ , et il n'est pas nécessaire de tamponner le milieu. Seuls des milieux acides peuvent d'ailleurs être tamponnés efficacement sans inconvénient. Les flagellés ne supportent pas une concentration de phosphate M/25 à  $p_H$  7.0 alors qu'ils se développent à  $p_H$  4.6 dans des milieux renfermant des phosphates à concentration M/3.5.

 ${\bf TABLEAU~I}$  variations de la résistance au phosphate en relation avec le  ${\bf p_H}$ 

| Après 2 jours |     |                   |                                    |                                                         | Après 6 jours                                                        |                                                       |                                                       | Après 4 jours                                         |                                                       |
|---------------|-----|-------------------|------------------------------------|---------------------------------------------------------|----------------------------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------|
| 7.4           | 7.2 | 7.0               | 6.6                                | 4.6                                                     | 7.4                                                                  | 7.2                                                   | 7.0                                                   | 6.6                                                   | 4.6                                                   |
| ++            | ++  | ++                | ++                                 | ++                                                      |                                                                      |                                                       |                                                       |                                                       |                                                       |
| 0             | О   | 土                 | i ' '                              |                                                         | ++                                                                   | ++                                                    | ++                                                    |                                                       |                                                       |
| 0             | 0   | 0                 | ± .                                | ++                                                      | 0                                                                    | О                                                     | +                                                     | ,                                                     |                                                       |
|               |     | 0                 | 0                                  | ++                                                      |                                                                      |                                                       | O                                                     | +                                                     |                                                       |
|               |     |                   |                                    | + +                                                     |                                                                      |                                                       |                                                       |                                                       | ++                                                    |
|               | ++  | 7·4 7·2 ++ ++ 0 0 | 7.4 7.2 7.0<br>++ ++ ++<br>• • • ± | 7.4 7.2 7.0 6.6<br>++ ++ ++ ++ ++<br>0 0 0 ±<br>0 0 0 ± | 7.4 7.2 7.0 6.6 4.6  ++ ++ ++ ++ ++ ++  o o o ±  o o ±  o o ++  ++ ± | $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ |

- ++ Culture abondante, plus de 1 000 flagellés/μl
  - + 200 à 1000 flagellés/µl
  - ± 1 à 20 flagellés/μl
  - o moins de 1 flagellé/ul

Les phosphates utilisés sont tous des phosphates R.A.L. ou Merck, qualité Sörensen ou "pour analyse".

L'ensemencement est effectué avec 20 ml d'une culture jeune dans des ballons renfermant 4 litres de milieu aérés par barbotage d'air et maintenus à 24°. Le barbotage doit être ménagé au début afin de ne pas diminuer trop la tension de CO<sub>2</sub>. Dans ces conditions, on obtient en 3 jours des cultures très abondantes. Celles-ci sont centrifugées dans une centrifugeuse Sharpless. A grande vitesse, les flagellés éclatent. La pâte blanchâtre est broyée avec du sable fin lavé. Le broyat est mis, suivant les cas, en suspension dans un tampon de phosphate M/100 ou de citrate M/50 de pH 7.0. Une première centrifugation à faible vitesse élimine avec le culot les grains d'amidon et des débris cellulaires. Le liquide trouble qui surnage est centrifugé à 12000 tours dans une centrifugeuse angulaire et le culot remis en suspension dans un tampon.

# MISE EN ÉVIDENCE D'UNE PHOSPHORYLASE

Les préparations enzymatiques sont additionnées d'un tampon de phosphate et d'amidon soluble. La concentration en phosphate est donnée dans le Tableau II. La concentration finale en amidon est de 2 à 5 mg/ml. On constitue un témoin sans amidon. Les tubes additionnés de toluène sont placés au bain-marie à 24°. Après traitement par l'acide trichloracétique, le phosphate minéral est dosé par la méthode de Fiske et Subbarow<sup>5</sup>. Le Tableau II montre les résultats de quelques expériences. On voit qu'il y a disparition du phosphate minéral en présence d'amidon.

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TABLEAU II DISPARITION DU PHOSPHORE MINÉRAL EN PRÉSENCE D'AMIDON

|                 | A    | В    | С    |
|-----------------|------|------|------|
| Enzyme témoin*  | 47·5 | 58.5 | 49·5 |
| Enzyme + amidon | 37·5 | 52.5 | 39   |

P minéral en  $\mu$ mol/ml.

### RECHERCHE DU GLUCOSE-I-PHOSPHATE

Nous avons donc cherché à mettre en évidence le glucose-I-phosphate. Il n'est pas possible d'éliminer des préparations brutes le phosphate minéral par le réactif ammoniaco-magnésien car le glucose-I-phosphate est coprécipité.

Nous avons donc utilisé la technique de Le Page et Umbreit, variante comportant la précipitation par l'éthanol, au premier stade, des sels de Ba. Si les opérations sont répétées, la plus grande partie du P minéral est éliminée. Le surnageant, après la dernière centrifugation destinée à éliminer le sulfate de Baryum est amené à p<sub>H</sub> 8.2 avec de la potasse et le sel de K de l'acide glucose-I-phosphorique précipité par l'alcool est séché sous vide.

L'hydrolyse par ClH M à 100° (et non M/10 ainsi qu'il a été imprimé par erreur dans notre note préliminaire) libère du phosphate minéral. L'hydrolyse est complète en 7 minutes. Elle libère aussi un sucre réducteur qui a été identifié au glucose par la forme cristalline de son osazone et aussi par l'action spécifique de la glucose-oxydase (notatine). Le dosage a été effectué par la méthode de Keilin et Hartree<sup>6</sup> à la notatine et par la méthode de Somogyi. Le tableau suivant montre que les deux techniques donnent des résultats identiques et que le glucide et le phosphate sont en quantités sensiblement équi-moléculaires.

TABLEAU III dosage du glucose et du phosphore dans deux hydrolysats  $(\mu \text{mol/ml})$ 

|                                                                      | A            | В    |
|----------------------------------------------------------------------|--------------|------|
| Glucose: méthode de Somogyi<br>Glucose: méthode de Keilin et Hartree | 2.05<br>2.04 | 13.7 |
| P minéral*                                                           | 1.86         | 12.8 |

<sup>\*</sup> Après soustraction du P minéral trouvé avant hydrolyse: A 0.15; B 1.7 μmol.

Le composé isolé à partir des préparations présente donc les propriétés suivantes: sels de Ba soluble à  $p_H$  8.2 et précipité par 4 vol. d'alcool. Pas de précipitation par le réactif ammoniaco-magnésien (après purification). Hydrolyse complète en 7 minutes à 100° par ClH M. Présence de glucose et de phosphate en quantités équimoléculaires. Il s'agit donc bien de glucose-1-phosphate.

En présence de glucose-1-phosphate (préparé avec la phosphorylase de la pomme de terre suivant la technique de Hanes) et de dextrine comme amorce, les préparations

<sup>\*</sup> La teneur en P minéral dans les préparations témoins est identique au départ et à la fin de l'expérience.

enzymatiques libèrent du phosphate minéral. Les préparations n'ayant pu être débarrassées des traces d'amylase, la synthèse d'amidon n'a pu être mise en évidence. Notons aussi qu'il n'apparaît pas de sucres réducteurs au cours de la libération du P minéral. Il nous paraît fort vraisemblable que l'action de la phosphorylase de *Polytomella* comme celle des phosphorylases classiques étudiées par W. Kiessling<sup>7</sup>, C. S. Hanes<sup>8</sup> et par C. F. Cori, G. Schmidt et G. T. Cori<sup>9</sup> est réversible.

### ESSAI DE PURIFICATION DE L'ENZYME

- a) La préparation est traitée par le sulfate d'ammonium au tiers de saturation. Le surnageant reste actif. Par contre, l'activité passe dans le culot après précipitation par le sulfate d'ammonium à demi-saturation.
- b) Si la préparation est centrifugée dans une centrifugeuse angulaire de Servall à 12000 tours par minute pendant 10 minutes, la fraction active est dans le culot. L'enzyme est lié à des granules qui sont visibles au microscope, mais que nous n'avons pas identifiés.

Cette centrifugation élimine la plus grande partie d'une amylase que nous n'avons cependant pas pu, même après centrifugations répétées, éliminer des granules contenant la phosphorylase. D'autres essais de purification n'ont pas été tentés.

# REMARQUES SUR L'AMYLASE

Les préparations d'amylase dans un tampon citrate M/20 ne donnent pas de sucre réducteur en 2 h aux dépens de l'amidon, mais uniquement des dextrines. Par contre, les préparations maintenues 24 h sous toluène en l'absence de phosphate minéral montrent une légère activité réductrice. Le sucre, qui est vraisemblablement du maltose, n'a pas été identifié. On sait que le maltose n'est pas utilisé par *Polytomella coeca*. Si donc la, ou les amylases intervenaient seules dans l'utilisation de l'amidon, leur action aboutirait à un glucide qui serait perdu pour les flagellés en culture bactériologiquement pure. Il est fort probable que l'amylase, ou des amylases, interviennent dans les premiers stades de l'utilisation des grains d'amidon, et que les dextrines produites au début de l'attaque sont phosphorylées et donnent du glucose-1-phosphate avant que le stade glucide réducteur ne soit atteint.

# DISCUSSION

On connaît jusqu'ici deux voies de biosynthèse de l'amidon: par la phosphorylase classique (C. Hanes, C. et G. Cori) et par l'amylomaltase (J. Monod et A. M. Torriani<sup>10</sup>). Ces deux enzymes représentent d'ailleurs conformément aux idées exprimées par Doudoroff, Barker et Hassid<sup>11</sup> et par A. M. Torriani et J. Monod<sup>12</sup> des transglucosidases.

Le défaut de l'utilisation du maltose et des autres disaccharides permet d'exclure l'hypothèse d'une synthèse de l'amidon chez *Polytomella* par une amylomaltase ou par un enzyme du même type. L'existence d'une phosphorylase suffit à rendre compte de la synthèse du polysaccharide.

Admettons que cette phosphorylase soit responsable de la synthèse de l'amidon chez le flagellé. Deux questions restent posées.

- I. Pourquoi les flagellés sont-ils incapables d'utiliser les glucides et, en particulier, le glucose? Si les flagellés possédaient une hexokinase et une phosphoglucomutase, ils seraient bien entendu capables de synthétiser le glucose-I-phosphate. L'absence de ces deux enzymes, ou d'un seul d'entre eux, suffit à expliquer le défaut d'utilisation du glucose. Nous avons en tous cas constaté que l'hexose-diphosphate mis en présence de préparations enzymatiques du flagellé n'est pas attaqué.
- 2. Comment les flagellés synthétisent-ils le glucose-I-phosphate? Cette question est actuellement à l'étude. L'hypothèse la plus simple est celle d'une synthèse par condensation aldolique sous l'influence d'un enzyme, d'acide dioxyacétone-phosphorique et de p-aldéhyde-glycérique. L'existence de cette réaction chez les levures a été démontrée par les recherches d'Otto Meyerhof<sup>13</sup>.

Quoi qu'il en soit, le flagellé *Polytomella cocca*, comme beaucoup de flagellés avec ou sans chlorophylle, synthétise de l'amidon et est incapable d'utiliser les glucides. Le glucose n'apparaît donc pas comme un métabolite intermédiaire obligé entre les aliments carbonés minéraux ou organiques et les polysaccharides. Des organismes peuvent synthétiser l'amidon et l'utiliser sans que le glucose apparaisse dans ce cycle autrement que sous forme phosphorylée.

# RÉSUMÉ

- ı. Le flagellé Polytomella coeca synthétise et utilise l'amidon. Ce flagellé est incapable d'utiliser les glucides pour son alimentation carbonée.
- 2. Le flagellé possède une phosphorylase: du glucose-I-phosphate a été isolé à partir de préparations enzymatiques additionnées d'amidon soluble et de phosphate minéral.
  - 3. Le problème de la synthèse du glucose-1-phosphate n'a pas été résolu.
- 4. Des organismes peuvent synthétiser l'amidon et l'utiliser sans que le glucose apparaisse dans ce cycle autrement que sous forme phosphorylée.

## SUMMARY

- 1. The flagellate  $Polytomella\ coeca$  synthesizes and utilizes starch. This flagellate is unable to utilize the sugars for its carbon-nutrition.
- 2. The flagellate contains a phosphorylase. Glucose-I-phosphate has been isolated from enzyme preparations to which soluble starch and mineral phosphate were added.
  - 3. The problem of the synthesis of glucose-I-phosphate has not been solved.
- 4. Organisms exist, which can synthesize and utilise starch, glucose appearing in the cycle only in phosphorylated form.

### ZUSAMMENFASSUNG

- 1. Der Flagellat *Polytomella coeca* baut Stärke auf und verwendet sie. Dieser Flagellat ist unfähig, die Zucker für seine Kohlenstoff-Nahrung zu verwenden.
- 2. Der Flagellat enthält eine Phosphorylase: Glucose-1-phosphat wurde aus Enzym-Präparaten isoliert, zu denen lösliche Stärke und mineralisches Phosphat zugegeben worden waren.
  - 3. Das Problem der Synthese des Glucose-1-phosphats ist noch ungelöst.
- 4. Es gibt Organismen, welche Stärke aufbauen und abbauen können, ohne dass Glucose in diesem Zyklus erscheint, ausser in phosphorylierter Form.

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# INHIBITION OF THE METABOLISM OF NUCLEATED RED CELLS BY INTRACELLUAR IONS AND ITS RELATION TO INTRACELLULAR STRUCTURAL FACTORS

by

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#### INTRODUCTION

The fundamental investigations of Meyerhof, Embden, Warburg, Cori and others on the anaerobic metabolism of the skeletal muscle, yeast and blood cells, and the discovery of the role of dicarboxylic and tricarboxylic acids in the oxidative metabolism of animal cells by Szent-Györgi and Krebs and of the mechanism of the hydrogen transfer to oxygen by Keilin and Warburg laid the foundations of our knowledge of the nature of chemical reactions providing the energy for cell activities. Meyerhof's work elucidated the correlation between certain oxidative and anaerobic enzyme reactions and certain phases of muscle activity. In general, however, our knowledge of the integration of enzyme reactions involved in aerobic metabolism into the organisation of the cell and its mechanism is rather inadequate.

The cell metabolism is not a static phenomenon. Any increase in cell activity following stimulation is accompanied by a very considerable increase of the oxidative cell metabolism. The latter goes on mainly at the expense of glucose taken up from the environment or glycogen present in the cell. There is some evidence scattered in the literature that the mechanism of this part of the oxidative metabolism of sugar, which appears after stimulation may not be completely identical that with of the oxidative metabolism of the resting cell. This evidence was obtained from the study of the metabolism of cells stimulated in vitro. In 1936 Deutsch and Raper<sup>1</sup> made the important observation that slices of glandular tissue (salivary gland, pancreas, liver) increase their O<sub>2</sub> uptake several times, when treated with certain hormones like acetyl choline, adrenaline and secretin, which in vivo stimulate the specific activities of those glands. Specific pharmacological stimulants of glands like pilocarpine showed the same effect. The increase is temporary, lasting about 30-60 minutes. It can, however, repeatedly be fully reproduced by a new dose of a stimulant some times after the preceding stimulation. Adrenaline provokes the increase in respiration only with salivary glands which can be physiologically stimulated by the sympathetic and adrenaline.

This fact, the reproducibility of the metabolic response to stimulants after a period of recovery and its temporary character, strongly suggest that this metabolic process

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in vitro is essentially with the metabolic response to stimulation in vivo. This is further borne out by observations of Brock, Druckerey and Herken² who confirmed the findings of Deutsch and Raper. They calculated the metabolic turnover of the whole salivary gland from the values obtained in vitro on slices and found after stimulation values which agreed well with values obtained by Barcroft and Peper³ on the salivary gland stimulated in vivo by chorda tympani. They found, furthermore, that the "stimulation metabolism", as they call the metabolic response of tissue slices to stimulants, depends on the ionic equilibrium in the Ringer solution in which the slices are suspended. Complete removal of the Ca from the Ringer suppresses completely the stimulation response, which can be restored by the subsequent addition of Ca. The removal of K ions does not suppress the first response but prevents the recovery. The ionic equilibrium in the medium is essential for the structural integrity of the cell or at least its surface membrane. It is therefore clear that the stimulation response requires the integrity of the cell structure and cannot be a consequence of injury and structural disintegration.

The stimulation metabolism shows two significant features as compared with the basic or rest metabolism: I. the latter has a R.Q. below I while the excess respiration after stimulation has a R.Q. of I, indicating a pure carbohydrate metabolism; 2. the increase in  $O_2$  uptake is always paradoxically accompanied by a production of free acids, of which at least half was shown by Deutsch and Raper to be lactic acid<sup>1</sup>. Brock, Druckerey, and Herken<sup>4</sup> have shown that this production of acid does not occur when K ions are removed from the surrounding medium, although the increase in respiration appears unchanged in size after the first stimulus.

The characteristic metabolism response to hormonal or pharmacological stimuli is by no means a peculiarity of glandular tissues. The increased respiration of the sea urchin egg after fertilization shows all the characteristic properties of the stimulation metabolism of glands<sup>5</sup>. The production of free acid in this case was found by Runnstrom, although the nature of the acid was not definitely established. As in glands there is also a marked difference in the sensitivity towards HCN between the respiration of the unfertilized and that of the fertilized egg. And according to Brock et al. a hormonal extract of the anterior pituitary which influences the division of the egg provokes the same characteristic metabolic response in it as fertilization. This cannot be obtained with extracts which do not influence the cleavage of the egg.

Finally a similar metabolic response was observed in 1937 by GOTTDENKER AND MARCHI<sup>6</sup> on mammalian heart lung preparations. They found that adrenaline, which is a heart stimulant, increased the O<sub>2</sub> uptake of these preparations and at the same time provoked an intensive lactic acid production.

The fact that the increased respiration in stimulated tissue slices goes on at the expense of carbohydrates and is accompanied by formation of lactic acid only under physiological conditions of the medium suggests a certain interpretation of the mechanism of this metabolic phenomenon. The anaerobic glycolysis of the glands is completely suppressed by the basic respiration due to the Pasteur effect. Any factor leading to a deteriorization of the structural integrity of the cell tends to provoke an aerobic glycolysis. This is the case for instance with liver or brain slices when K is removed from the medium. The aerobic glycolysis accompanying the stimulation response differs in this respect fundamentally in being dependent on the presence of K ions in the medium and is suppressed completely after their elimination. This indicates clearly that aerobic glycolysis of stimulation is not due to structural damage or increase of per-

meability, but to a specific coupling between the oxidative breakdown of sugar and glycolysis. Now it is reasonable to assume that phosphorylation of glucose to hexosediphosphate constitutes the first steps in glycolysis. Any coupling between glycolysis and respiration therefore will consist primarily in a coupling between certain oxidative processes and phosphorylation of glucose. It is well known that the oxidation of pyruvic acid in the Krebs cycle is coupled with an intensive phosphorylation of glucose and adenylic acid (to ATP). Certain individual enzyme reactions in the Krebs cycle, like oxidation of the succinic and a-ketoglutaric acid, have been shown to be coupled with phosphorylation of glucose and adenylic acid<sup>7</sup>. Quite recently the same was shown for the electron transfer from dihydrocozymase to the cytochrome system<sup>8</sup>. Ochoa<sup>9</sup> has shown for heart muscle extracts that complete oxidation of one molecule of pyruvate can be coupled with the phosphorylation of 9 molecules of glucose to hexosediphosphate. The oxidation of I molecule of glucose over the Krebs cycle therefore can phosphorylate 18 molecules of glucose. That this excess phosphorylation does not appear in resting cells must be ascribed to the coupling of the phosphorylation of glucose with oxidative processes in such a way that the speed of these processes does not exceed the maximal speed of oxidation of pyruvate. If the KREBS cycle is operating and these controls are eliminated, aerobic glycolysis or accumulation of hexosephosphate must result. All these considerations suggest that the metabolic response to stimulation in organs may be due to a release or increase of the activity of the tricarboxylic acid system and accompanying phosphorylation. In the metabolism of resting cells this system may play only a minor role or be lacking altogether. This view appears supported by the fact that cells like embryonic and tumor cells, et al., which according to Brock do not show any stimulation response in vitro, show only very weak activity of enzymes belonging to the tricarboxylic acid system.

Turning to the consideration of possible mechanisms involved in the release of the metabolic response to stimulation we must keep in mind that every cell responds to stimulation by the electric current essentially in the same way as to that by nervous impulses or hormonal and pharmacological stimuli. The primary effect of the electric stimulus consists in shifts of intracellular ions. It is generally assumed that such shifts, with consecutive accumulation of certain ions on intracellular membranes, are responsible for the functional response to stimulation. It may reasonably be assumed that such shifts of intracellular ions are also instrumental in provoking the metabolic response. As the latter can be more protracted than the functional response the effects of ionic shifts must be more complex in this case and consist in a chain of reactions released by the primary shift. The ions could exert their influence either directly on enzymes involved in the stimulation metabolism or indirectly by changing the permeability of intracellular membranes and thus facilitating the access of substrates to certain enzymes.

It was observed recently that hemolysates of nucleated red cells of pigeon glycolyse only in presence of oxygen. This aerobic glycolysis disappears in presence of M/500 NaCN. It was further found that all intracellular polyvalent ions like Mg, Ca, orthophosphate, ribonucleate inhibit the aerobic glycolysis in physiological concentration. Colowick, Kalckar and Corit found in 1941 a similar obligatorily aerobic glycolysis in kidney extracts and showed that it is dependent upon the oxidation of succinic acid. As it was known that nucleated red cells are able to oxidise pyruvic acid to CO<sub>2</sub> and that their respiration is coupled with the synthesis of ATP it seemed reasonable to assume that the aerobic glycolysis in hemolysates of these cells is the result of the coupling

of phosphorylation of glucose with the oxidative processes of the Krebs cycle. The inhibitory effects of ions on the aerobic glycolysis suggested that we are here in presence of an enzymatic system displaying this sensitivity towards ions which underlies the mechanism of the metabolic response to cell stimulation.

The possible general physiological significance of this phenomenon invites closer investigation of its mechanism. The present report deals with experiments in this direction.

#### EXPERIMENTAL

#### A. Preparation of the material

Red blood cells of pigeons were used for the experiments. The animals were kept fasting for at least 12 hours preceding the bleeding, which was carried out by cutting the throat on one side after removal of feathers. The blood was caught in a dish containing 0.3 ml of 3.6% sodium citrate. It was centrifuged and the upper stratum of the sediment, containing the white cells, was removed as far as possible by pipetting. The remaining red cells were first washed twice with a fivefold volume of a mixture of 1 part 3.6% sodium citrate and 9 parts of 0.9% NaCl and then 3 times with the NaCl solution. The washed cells were hemolyzed by adding 1.5 parts of distilled water to 1 part of cells. The pH of these hemolysates was found to vary between 7.25 and 7.15. As it was intended to investigated the effect of salts on the metabolism of the hemolysate it was not possible to use buffers in our experiments and we had to rely for the stabilization of pH during the experimental period on the considerable buffering capacity of hemoglobin. Orienting experiments, however, showed that the shift of pH due to acid formation during 4 hours at 25° did not exceed 0.2. The optimal pH for the aerobic metabolism was found to be about 6.8. In most of our experiments the pH at time 0 was therefore that of the original hemolysate or slightly lower, i.e., 6.9–7.0. The latter was obtained by adding an appropriate amount of diluted HCl to the water used for hemolysis.

#### B. Analytical methods

In a certain number of experiments a complete balance of O2 uptake, CO2 production, and glucose consumption was carried out. In these and most of the other experiments the total volume of either water or of respective solutions added to the hemolysate was 0.2 ml per 1 ml of the original hemolysate. The final dilution of the original cell suspension was therefore threefold. All experiments were done at 25° and lasted as a rule four hours. The O2 uptake was measured on 2 ml of the hemolysate in standard Barcroft-Warburg manometers with absorption of CO2 and NH3. This shifted the  $p_H$  of the hemolysate no more than 0.1 to the alkaline side.  $CO_2$  production was determined by the direct method. To account for the retention of CO<sub>2</sub> by the hemolysate the manometer in which CO<sub>2</sub> was not absorbed contained in a second sidearm 0.4 ml of diluted H<sub>2</sub>SO<sub>4</sub>. At the end of the experiment the acid was tipped in from the sidearm into the hemolysate. The  $m p_H$  of the latter was then shifted below 4. The hemolysate became very viscous at this  $p_{
m H}$  but came into the equilibrium with the gas phase after about 30 minutes. As the hemolysate contained from the beginning a certain amount of bound CO2 the same procedure was carried out on a sample of the hemolysate at time o. The difference of the increase in gas volume after addition of acid in the two samples gave the amount of CO2 produced by oxidation and retained by the hemolysate. At the end of the experiment 1 ml of the hemolysate was pipetted out of the manometer vessels, deproteinezed with 4 ml of 7.5% trichloracetic acid. The centrifugate served for the determination of lactic acid and glucose and phosphate fractions. The lactic acid determination was carried out by the procedure of Barker and Summerson<sup>12</sup>, glucose by the new spectrophotometric micromethod of Dische, Shettles and Osnos<sup>13</sup> based on a specific reaction of hexoses with cysteine in H<sub>2</sub>SO<sub>4</sub>. In this reaction fructose gives only 12% more absorption than the equivalent of glucose, so that the phosphorylation of a small amount of the latter to Harden-Young ester will not influence significantly the accuracy of the determination. In some experiments we tested for this ester and triosephosphate by a new highly sensitive reaction with carbazole, which allows the determination of fructose and triosephosphate in the same sample. Inorganic and the labile phosphate were determined by the FISKE-Subbarow method in the modification of King, ribose and adenosine-5-phosphate by the orcinol reaction.

## C. Results

In a first series of experiments the aerobic metabolism of the hemolysate was examined to obtain information about the nature of enzyme reactions involved in this *References p. 292*.

metabolism. In a second series the influence of various cations and anions on those reactions was investigated.

# 1. The aerobic metabolism in the hemolysate

- a) O<sub>2</sub> uptake, lactic acid formation in the hemolysate in absence of glucose. The hemolysate to which 0.2 ml of liquid per ml was added shows a marked respiration which varied in our experiments between 19 and 92 cmm per 1 ml and 4 hours. The respiration is in general much higher during the first hour and drops afterwards to a lower but constant level. The R.Q. varies considerably between 0.82 and I (Table I). The erythrocytes contain very little hexoses soluble in trichloracetic acid. Less than I  $\gamma/ml$  of hexose (calculated as glucose) was found in the hemolysate. This amount does not change during the 4 hours of the experiment. On the other hand there is a considerable decrease in the amount of adenosine-5-phosphate. In experiment VI (Table I) 84 y/ml of this compound, corresponding to 35  $\gamma$ /ml pentose, disappeared in 4 hours. If all of this pentose had been oxidized to CO<sub>2</sub> half of the total O<sub>2</sub> uptake in this experiment would be accounted for. The breakdown of adenosine-5-phosphate can be explained by the fact that it is formed in the hemolysate by the ATPase and dephosphorylated to adenosine which, as was shown for human erythrocytes, can be split, with phosphorylation, to form triosephosphate and hexosediphosphate. One part of the respiration of the hemolysate in absence of glucose must be due to the oxidation of either fat or protein. The hemolysate contains from the beginning very small amounts of lactic acid (about 5  $\gamma$ /ml). In some cases small amounts of this acid are formed during incubation, but not more than about 5  $\gamma$ /ml.
- b) The tricarboxylic acid cycle in the hemolysate. The presence of this enzyme system in the hemolysate can be demonstrated after addition of citrate or one of the dicarboxylic acids metabolised by the system. When M/I 200 of succinic, fumaric, malic, oxaloacetic, citric and α-ketoglutaric acid is added the O2 uptake increases considerably (Table I). In presence of ketoglutaric and citric acid much more than in that of other acids this additional O2 uptake increases with the concentration of the acid. It is about twice as great in presence of M/600 succinate than of M/1200. At the same time lactic acid is formed in significant amounts. This increases with the concentration of succinate or malate. The amount of lactic acid varies with the nature of the acid in the following sense: malate, fumarate > succinate >  $\alpha$ -ketoglutarate > citrate. This can be explained by the assumption that oxaloacetic is formed from malic acid, with reduction of coenzyme I to dihydrocoenzyme I. One part of the oxaloacetic acid is decarboxylated to pyruvate and CO<sub>2</sub>. As the cytochrome system is not able to oxidize dihydrocozymase rapidly enough, one part of it reduces pyruvate to lactate. The same sequence of reactions was observed by E. A. Evans<sup>14</sup> in liver extracts. As the increase in succinate increases the O2 uptake as well as lactic acid formation the cytochrome system apparently competes with the pyruvate for dihydrocozymase. Thus the fact that lactic is formed from citrate indicates that the whole series of reactions from citrate to oxaloacetates goes on in the hemolysate. Pyruvic acid also increases the respiration and lactic acid formation, though less than any one of the polycarboxylic acids, and the increase is observed only during the last 3 hours of the 4 hour period.

# 2. Aerobic metabolism in presence of glucose

When 50 mg % of glucose is added to the hemolysate it is broken down at a rate References p. 292.

#### TABLE I

Influence of MgCl $_2$  M/250 ,of pyruvic, citric and dicarboxylic acids of the Krebs cycle on the  $\rm O_2$  consumption of the hemolysate in presence and absence of glucose and on aerobic glycolysis. Time of exp.: 4 h the bracketed values represent the  $\rm O_2$  uptake in the last 3 h

|             |                                                                       | $O_2$                                          | uptake in 1/ml o                          | f hemolysate                      | in $\mu$ l                   | Aerobic gly                            | colysis      |
|-------------|-----------------------------------------------------------------------|------------------------------------------------|-------------------------------------------|-----------------------------------|------------------------------|----------------------------------------|--------------|
| Exp.<br>No. | Substance<br>added                                                    | by<br>hemolysate<br>itself                     | change<br>%                               | glucose<br>in the<br>hemolysate   | change<br>0/<br>/0           | γ lactic acid<br>γ/ml of<br>hemolysate | change       |
| I           | o<br>a. MgCl <sub>2</sub> M/250<br>b. Na succinate                    | 43·5 (31)<br>54 (32·4)                         | + 24 (+ 5)                                | 8.2 (3.0)<br>15 (12.5)            | + 84 (+ 320)                 | 168<br>245                             | + 46         |
|             | M/1540<br>a + b                                                       | 52 (35.6)<br>75 (47.5)                         | + 20 (+ 16)<br>+ 70 (+ 53)                |                                   |                              | 218<br>259                             | + 30<br>+ 54 |
| II          | o<br>MgCl <sub>2</sub> M/250<br>Na succinate<br>M/770                 | 34.2 (23.8)<br>45 (28.5)<br>60.2 (34)          | + 31.6 (+ 19.9)<br>+ 76 (+ 43)            | 8.3 (2.4)<br>13.9 (11)            | + 67 (+ 360)                 | 220<br>265                             | + 21         |
| III         | o<br>MgCl <sub>2</sub> M/250<br>Na Pyruvate<br>M/1200                 | 29 (18.7)<br>34.3 (25.5)<br>21.7 (21.7)        | + 18 (+ 36)<br>- 25 (+ 16)                | 6.7 (8)<br>12.6 (10.1)<br>14 (5)  | + 88 (+ 26)<br>+ 10.9 (-37)  | 139<br>185<br>156                      | + 33<br>+ 12 |
| IV          | 0 Na succinate M/1200 Na citrate Na /1200 Na α-keto glutarate M/1200  | 44·3 (27)<br>54 (37)<br>60 (41)<br>67 (42·5)   | + 22 (+ 37)<br>+ 35 (+ 51)<br>+ 52 (+ 57) |                                   |                              |                                        |              |
| V           | o Na succinate M/1 200 Na citrate M/1 200 Na a-keto glutarate M/1 200 | 32.4 (21)<br>49 (34.3)<br>55.4 (41)<br>64 (43) | + 51 (63)<br>+ 71 (+ 95)<br>+ 100 (+ 102) |                                   |                              |                                        |              |
| VI          | o<br>Na pyruvate<br>M/1 200                                           | 45.6 (30.1)<br>48 (34.6)                       | + 5.3 (+ 15)                              | 6 (10.5)<br>o (o)                 | —100 (—100)                  | 262<br>258                             | — I.5        |
| VII         | 0                                                                     | 35 (19.1)                                      |                                           | 17.5 (12.3)                       |                              | 164                                    |              |
|             | Na succinate M/I 200                                                  | 55 (36.1)                                      | + 57 (+ 89)                               | 13.3 (12.4)                       | — 24 (+ I)                   | 218                                    | + 33         |
|             | Na pyruvate<br>M/1 200                                                | 46.5 (29.9)                                    | + 33 (+ 55)                               | 6.7 (6.1)                         | — 62 (— 50)                  | 169                                    | + 3          |
| VIII        | o<br>Na pyruvate                                                      | 75.6 (38.6)                                    |                                           | 8.6 (20.7)                        |                              | 276                                    |              |
|             | M/1 200                                                               | 78.7 (47)                                      | + 4.1 (+ 22)                              | 14.3 (8.9)                        | + 66 ( 58)                   | 246                                    | - 11         |
| IX          | 0<br>NaCN M/500<br>NaCN (M/250                                        | 27.8 (18.7)<br>2.5 (2.9)<br>0 (0)              | — 91 (— 87)<br>— 100 (— 100)              | 13.3 (10.3)<br>3.3 (1.2)<br>0 (0) | - 75 (- 88)<br>- 100 (- 100) | 159                                    | <b>- 92</b>  |

of 75–150  $\gamma$ /ml per hour. The  $O_2$  consumption increases at the same time considerably by 13–50% in 4 hours. At the same time an intensive aerobic glycolysis and sometimes esterification of inorganic P to difficultly hydrolyzable esters is observed. Up to 260  $\gamma$ /ml of lactic is produced in 4 hours. The rate of  $O_2$  consumption during the first hour is different from the rate in the following 3 hours during which it remains almost constant. The rate of glycolysis is in general smaller during the first hour than later. If we assume that the additional  $O_2$  consumption in presence of glucose is due to the total oxidation of the latter and calculate the total breakdown of glucose by oxidation and glycolysis the latter turns out to be considerably smaller than the amount of glucose which really disappeared. The R.Q. of the additional respiration due to glucose is only about 0.7 (Table VI). The discrepancy between the observed values and those calculated for glucose which disappears indicates that only one part of it is completely oxidized while another part is oxidized either to phosphogluconic or pyruvic acid.

# 3. The coupling between aerobic glycolysis and respiration

The glycolysis of the hemolysate is obligatorily aerobic and disappears almost completely when the oxidation processes in the hemolysate are suppressed either by inhibitors or by elimination of O2. Thus NaCN at M/250 almost completely suppresses the glycolysis and 90% of the total  $O_2$  consumption. (Table I) Further increase of the concentration does not have any significant effect. The small residual glycolysis amounts to only a few per cent of the total and is probably due to the leucocytes which were not removed. The leucocytes which are siphoned off in the beginning of the blood washing display in fact a powerful anaerobic glycolysis which is partly suppressed in aerobiosis. That the effect of cyanide on glycolysis is due to the blocking of respiration could be shown in experiments in which O was removed from the hemolysate. These were carried out in the following way. 4 ml of the hemolysate + 0.8 ml of 0.3% glucose solution were pipetted into a 500 ml flask which was closed by a ground stopper with stopcock. The flask was weighed and then evacuated first with a water pump. When the foaming of the fluid became too intense the evacuation was interrupted until the foam broke down and the evacuation then resumed until no more gas escaped. The evacuation was continued with the oil pump until a vacuum of about 1 mm Hg was obtained. The flask was then weighed again to determine the loss in water. The hemolysate was kept in vacuo for 4 hours at room temperature and then the flask opened, the evaporated water replaced and the hemolysate deproteinized simultaneously with a control, which stayed during the same period in presence of oxygen and one to which NaCN M/500 was added. The determination of lactic acid showed that the glycolysis was suppressed in the sample in vacuo, though not quite as far as in the sample with NaCN.

While suppression of the  $O_2$  consumption inhibits the glycolysis in our hemolysate any increase of  $O_2$  consumption after addition of pyruvate, citrate and dicarboxylic acids of the Krebs cycle is accompanied by a strong increase of glycolysis (Table II). If the final dilution of the hemolysate is no more than the threefold of the original volume of the suspension,  $\alpha$ -ketoglutarate is most effective, with succinate and fumarate following, and pyruvate the least effective. It was found for the succinate that the promoting effect on glycolysis increases with the concentration, as also does the  $O_2$  consumption.

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TABLE II  ${\rm Influence\ of\ pyruvate,\ citrate\ and\ dicarboxylic\ acids\ of\ the\ Krebs\ cycle\ on\ aerobic\ glycolysis\ in\ the\ hemolysate.\ time\ 2\ hours,\ t\ 25^\circ }$ 

| Experiment<br>No. | Substance added                                                                                                                              | mg lactic acid/ml<br>of hemolysate | Change %                      | РН  |
|-------------------|----------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------|-------------------------------|-----|
| I                 | $\begin{array}{c} \text{o} \\ \text{M/600 MgCl}_2 \\ \text{M/1 200 succinate} \\ \text{M/600 MgCl}_2 + \text{M/1 200 succinate} \end{array}$ | 46<br>73<br>96<br>131              | + 58<br>+ 109<br>+ 185        | 7.0 |
| ΙΪ                | o $M/600 \text{ MgCl}_2$ $M/1 200 \text{ succinate}$ $M/600 + M/1 200 \text{ succinate}$                                                     | 58<br>81<br>100<br>120             | + 40<br>+ 72<br>+ 108         | 7.0 |
| 111               | o M/1 200 succinate M/300 MgCl <sub>2</sub> M/1 200 Na pyruvate M/1 200 Na pyruvate + M/1 200 succinate                                      | 42<br>54<br>80<br>39·5<br>53       | + 29<br>+ 90<br>- 6<br>+ 29   | 7.0 |
| IV                | o<br>M/1 200 succinate<br>M/800 succinate                                                                                                    | 103<br>151<br>158                  | + 46<br>+ 53                  | 6.8 |
| V                 | o M/400 succinate M/400 $lpha$ -ketoglutarate                                                                                                | 35<br>47<br>44                     | + 34<br>+ 26                  | 7.1 |
| VI                | o<br>M/400 succinate<br>M/1000 pyruvate                                                                                                      | 7.5<br>29.5<br>15                  | + 300<br>+ 100                | 7.0 |
| VII               | o<br>M/1 200 succinate<br>M/800 succinate                                                                                                    | 48<br>123<br>132                   | + 156<br>+ 175                | 7.2 |
| VIII              | M/800 $a$ -ketoglutarate<br>M/1 200                                                                                                          | 168                                | + 250<br>+ 108                |     |
| IX                | O<br>M/450 succinate<br>M/450 malate                                                                                                         | 42<br>86<br>64                     | + 105<br>+ 57                 | 7.2 |
| X                 | o M/450 succinate M/900 succinate M/450 malate M/900 malate                                                                                  | 24<br>52<br>38<br>41<br>28         | + 116<br>+ 58<br>+ 70<br>+ 17 | 7.2 |

# 4. Influence of ions on the aerobic metabolism in presence and absence of glucose

Two different effects of ions on the aerobic metabolism in the hemolysate can be observed. The first is specific for magnesium ions and the second is common to all multivalent ions. In this second group, the nature and the charge of the ion is important for the intensity of the effect.

a) Magnesium. In concentrations up to  $M/200~MgCl_2$  increases the basic  $O_2$  consump-References p. 292.

tion in the hemolysate as well as the additional uptake in presence of glucose and the dicarboxylic acids. The increase ranges from 18 to 24% for the basic respiration and from 67 to 88% for the additional respiration due to glucose (Table I). At the same time there is an increase of the aerobic-glycolysis amounting to 21–46% of the original value (Table I, Exp. I–III). This effect of Mg reaches its maximum at M/200 to M/150. The additional  $\rm O_2$  uptake as well as the accompanying aerobic glycolysis are inhibited by M/500 NaCN to the same extent as is the case without addition of Mg.

- b) Univalent cations. When so much KCl is added to the hemolysate that the concentration of the added salt in the hemolysate becomes I/II M and the hemolysate therefore isotonic no inhibition of the basic  $O_2$  uptake with and without glucose can be observed. The aerobic glycolysis is in general somewhat decreased. In some cases, however, a decrease of 60% was observed. NaCl at the same concentration decreases the  $O_2$  uptake moderately and inhibits the aerobic glycolysis 33–50%. It must be noted that this concentration of Na ions cannot be considered any more as physiological. If the concentration of the added NaCl was only M/25 no significant inhibition of the  $O_2$  uptake or aerobic glycolysis could be observed. These observations indicate that Cl ions in physiological concentrations do not have any significant effect on the aerobic metabolism of the hemolysate.
- c) Calcium and other multivalent cations. When the concentration of Mg exceeds M/150 the aerobic glycolysis in the hemolysate begins te decline. At M/80 an inhibition of about 15-25% appears. This inhibitory effect is a property of all multivalent cations. (Table IV). Of all the cations investigated Ca shows the strongest inhibitory effect. M/1000-M/1500 shows almost complete inhibition of the aerobic glycolysis. Sr is almost as strong but Ba++, Ce+++ and La+++ are ten times weaker inhibitors. However, our figures merely correlate the strength of the inhibition with the overall concentration of the salt. The latter is almost identical with the concentration of the bivalent ions for the earth alkalis and rare earth but not for the other metals, the hydroxides of which possess low second dissociation constants. The ion Mn<sup>++</sup> and Cd<sup>++</sup> as such are, therefore, probably stronger inhibitors than Ca++. This however does not seem of any physiological significance. The inhibitory effect of Ca on the glycolysis is still perceptible at M/8000. After having ascertained that the inhibitory effect of Mg and Ca on glycolysis is related to their multivalence the effects on the O<sub>2</sub> consumption of those two as representatives of multivalent cations were studied. The basic O<sub>2</sub> consumption was inhibited 28-52% by Ca M/1000. The oxidation due to glucose, however, may completely disappear at this concentration while that of succinate and a-ketoglutarate is reduced to about the same extent as the basic respiration (Table III).
- d) Anions. All multivalent anions inhibit strongly the aerobic glycolysis (Table III). The importance of valency is more marked with anions than cations. The bivalent HPO<sub>4</sub>— and SO<sub>4</sub>— show a significant inhibition only at M/100 and M/50 respectively, while the tetravalent Fe(CN)—— at M/250, ribonucleate, diphosphoglycerate and inositolhexaphosphate strongly at M/1500, M/700 and M/1000 respectively. The nature of the ion plays, however, also a considerable role. The bivalent oxalate for example shows at M/1000 a stronger inhibition than malonate at M/200. The physiological polycarboxylic acids like succinate and citrate, which up to M/500 increase the aerobic glycolysis, inhibit at higher concentrations. At M/50 the inhibition is considerable with citrate. That multivalency is only one of the factors promoting the inhibitory effect on the metabolism is shown by the behaviour of the CNS— ion. While KCl at M/11 and

TABLE III effect of KCl, NaCl, MgCl $_2$  and of multivalent ions on the O $_2$  consumption by the hemolysate itself and by glucose, succinate,  $\alpha$ -ketoglutarate in the hemolysate. Time 4 hours

| Experi- | Cubatanaa addad                                    |                              | emol-<br>itself   | By gl                      | ucose             |                        | ccinate<br>1 200  |                  | eto glu-<br>M/1 200 |
|---------|----------------------------------------------------|------------------------------|-------------------|----------------------------|-------------------|------------------------|-------------------|------------------|---------------------|
| Ño.     | Substance added                                    | O <sub>2</sub><br>used       | Inhibi-<br>tion % | O <sub>2</sub><br>used     | Inhibi-<br>tion % | O <sub>2</sub><br>used | Inhibi-<br>tion % | ${\rm O_2}$ used | Inhibi-<br>tion %   |
| I       | Mg M/250<br>Mg M/250 + Ca M/2000                   | 54<br>48.5                   | 10                | 15<br>8.2                  | 45                |                        |                   |                  |                     |
| II      | Mg M/250<br>Mg M/250 + Ca M/1000                   | 45<br>21.5                   | 52                | 13.6                       | 93                |                        |                   |                  |                     |
| 111     | o<br>Ca M/1 000                                    | 92<br>50.6                   | 45                |                            |                   | 21.8<br>14.3           | 34                | 40.2<br>25.4     | 37                  |
| IV      | O<br>Ca M/1000<br>Mg M/250<br>Mg M/250 + Ca M/1000 | 45.6<br>24.7<br>45.6<br>23.6 | 46<br>48          | 5.9<br>0.3<br>21.3<br>11.5 | 95<br>46          |                        |                   |                  |                     |
| V       | о<br>Са М/1 000                                    | 35<br>18.9                   | 46                | 17.5<br>4.3                | 76                | 20<br>5.9              | 70                |                  |                     |
| VI      | o<br>Ca M/1 000                                    | 75.6<br>34                   | 55                | 8.7<br>4.1                 | 53                |                        |                   |                  |                     |
| VII     | o<br>Ca M/1000                                     | 32.1<br>23                   | 28                | 10.5                       | 100               |                        |                   |                  |                     |
| VIII    | o<br>KCl M/12                                      | 27.8<br>29.4                 | _                 | 13.4<br>14                 |                   |                        |                   |                  |                     |
| IX      | o<br>PO <sub>4</sub> M/500<br>Oxalate M/500        | 48<br>40.3<br>41.6           | 16<br>14          | 11<br>1<br>6.2             | 90<br>38          | 29.4<br>16.3           | 45                |                  |                     |
| X       | o<br>Oxalate M/500                                 | 44·3<br>29·5                 | 34                |                            |                   | 10.1<br>19             | _                 | 23.3<br>32.5     |                     |
| IX      | o<br>Oxalate M/250                                 | 3 <sup>2</sup> ·4<br>17.8    | 46                | 0.7                        | 93                | 16.8<br>11             | 34                | 31.4<br>32.6     | _                   |
| XII     | 0<br>Na <sub>2</sub> SO <sub>4</sub> M/2.4         | 60.2<br>47·4                 | 21                | 21<br>4.1                  | 80                | 19.2<br>16             | 17                | 29.5<br>26.6     | 10                  |
| XIII    | 0<br>NaCl M/12<br>NaCl M/25<br>KCl M/12            | 56.3<br>51.6<br>68.4<br>56.3 | 8                 |                            |                   |                        |                   |                  |                     |
| XIV     | o<br>Ribosenucleic acid M/1 500                    | 56<br>43.1                   | 23                | 14.2                       | 80                |                        |                   |                  |                     |

 $p_{\rm H}$  7.2 does not inhibit at all or only little, KCNS at the same concentration completely inhibits glycolysis (Table III).

The  $O_2$  consumption is suppressed by anions to about the same extent as glycolysis. References p. 292.

TABLE IV  $\\ \text{INHIBITION OF AEROBIC GLYCOLYSIS BY VARIOUS CATIONS AND ANIONS. TIME: EXPERIMENT I-X 4 HOURS, } \\ \text{EXPERIMENT XI 2 HOURS}$ 

| Experiment<br>No. | Substance added                                                                                                                                                                           | γ Lactic acid formed in<br>1 ml of hemolysate | Inhibition<br>%                  | Рн   |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|----------------------------------|------|
| I                 | O<br>HCN M/500<br>CaCl <sub>2</sub> M/1 000<br>BaCl <sub>2</sub> M/333<br>SO <sub>4</sub> M/333<br>MnCl <sub>2</sub> M/333<br>CaCl <sub>2</sub> M/333                                     | 62<br>11.2<br>11<br>20.7<br>18.6<br>11        | 82<br>82<br>66<br>70<br>82<br>82 | 7.25 |
| II                | o<br>SrCl <sub>2</sub> M/800<br>FeSO <sub>4</sub> M/333<br>CdSO <sub>4</sub> M/333<br>HCN M/500                                                                                           | 71.8<br>5<br>11.9<br>5.8<br>0.07<br>0.64      | 93<br>83<br>92<br>99             | 7.1  |
| III               | 2,3 diphosphoglycerate M/500<br>Inositol hexaphosphate M/700                                                                                                                              | 39<br>22.1                                    | 39<br>65.5                       | 7.2  |
| IV                | CaCl <sub>2</sub> M/4 000<br>Phosphate M/50<br>Na <sub>2</sub> SO <sub>4</sub> M/50<br>Na Citrate M/50                                                                                    | 37.2<br>19.4<br>28.3<br>18.4<br>5.2           | 48<br>23<br>50<br>86             |      |
| V                 | o<br>Ribosenucleic acid M/1600<br>Yeast adenylic acid M/400                                                                                                                               | 46.5<br>37.2<br>48                            | 20<br>3                          | 7.25 |
| VI                | o<br>CaCl <sub>2</sub> M/1 000<br>Na Succinate M/1 200<br>CaCl <sub>2</sub> M100 + Na Succinate M/1 200                                                                                   | 164<br>30<br>218<br>27                        | 82<br>83                         | 7    |
| VII               | o<br>NaCl 1/11<br>KCl 1/9                                                                                                                                                                 | 64<br>65<br>33                                | 48                               | 7.2  |
| VIII              | o<br>KCl 1/11<br>MgCl <sub>2</sub> M/250<br>MgCl <sub>2</sub> M/250 + KCl 1/11                                                                                                            | 36<br>35<br>45<br>37                          | 3                                | 7.2  |
| IX                | o<br>NaCl M/11<br>KCl M/11                                                                                                                                                                | 227<br>128<br>85                              | 43<br>63                         | 6.9  |
| х                 | o<br>KCl M/11<br>NaCl M/11<br>Ribonucleate M/1540                                                                                                                                         | 290<br>158<br>163<br>169                      | 45<br>43<br>42                   | 6.9  |
| XI                | o<br>M/300 MgCl <sub>2</sub><br>M/1 200 Na Succinate<br>M/1 000 CaCl <sub>2</sub><br>M/1 000 CaCl <sub>2</sub> + MgCl <sub>2</sub> M/300<br>M/1 000 CaCl <sub>2</sub> + M 1 200 Succinate | 103<br>177<br>151<br>34<br>13.6               | 97<br>87<br>85                   | 7.0  |

Different oxidation processes, however, are influenced to a very different degree. The oxidation of glucose suffers much more than the basic oxidation. M/480 sodium oxalate suppresses the additional respiration by glucose 80–100%, the basic only 0–15%. Essentially the same relation is valid for M/25 Na<sub>2</sub>SO<sub>4</sub>, M/50 Na phosphate and M/1700 Na ribonucleate. The oxidation of succinate is less suppressed than that of glucose but more so than that of  $\alpha$ -ketoglutarate and citrate.

e) Synergy between Mg and Ca and anions in their inhibitory effects. Effects of ions on colloidal particles are in general counteracted by ions of opposite charge if the effects are due to neutralization of electric charges. It is, therefore, surprising that inhibitory effects of anions on the metabolism of red cells are not eliminated or decreased, but on the contrary strongly enhanced by Mg and Ca (other multivalent cations

TABLE V Synergy between  ${\rm Mg^{++}}$  and  ${\rm Ca^{++}}$  and multivalent anions in their effects on the aerobic glycolysis of the hemolysate in presence of glucose. Temp. 25°. Time: exp. I–V 4 hours, exp. II 2 hours

| Experiment No. | Inhibitor                        | Lactic acid formed in mg/ml of hemolysate | Inhibition % | РН   |
|----------------|----------------------------------|-------------------------------------------|--------------|------|
| I              | a. o                             | 36                                        |              | 7.2  |
| _              | b. Na phosphate M/50             | 2 5                                       | 30           | •    |
|                | c. CaCl, M/4000                  | 34                                        | 6            |      |
|                | b. + c.                          | 4.3                                       | 88           |      |
|                | d. MgSO4 M/150                   | 38.5                                      |              |      |
|                | e. KCl M 1/10                    | 35                                        | 3<br>8       |      |
|                | d. + e.                          | 33                                        | 8            |      |
| 11             | a. o                             | 71                                        |              | 7.15 |
|                | b. CaCl <sub>2</sub> M/4 000     | 50                                        | 30           |      |
|                | c. Na phosphate M/50             | 55.4                                      | 22           |      |
|                | b. + c.                          | 28.3                                      | 60           |      |
| III            | a. o                             | 46.5                                      |              | 7.2  |
|                | b. CaCl <sub>2</sub> M/4000      | 34                                        | 27           |      |
|                | c. Inositol hexaphosphate M/1000 | 31                                        | 33           |      |
|                | b. + c.                          | 6.6                                       | 86           |      |
|                | d. $Na_2SO_4$ M/100              | 41.4                                      | II           |      |
|                | b. + d.                          | 9.2                                       | 8o           |      |
|                | e. MgCl <sub>2</sub> M/250       | 62                                        |              |      |
|                | e. + d.                          | 38.1                                      | 38           |      |
| IV             | a. o                             | 33                                        |              | 7.2  |
|                | b. phosphate M/50                | 41                                        | - 24         |      |
|                | c. MgCl <sub>2</sub> M/250       | 66                                        |              |      |
|                | b. + c.                          | 40                                        | 40           |      |
| V              | a. o                             | 202                                       |              | 7.25 |
|                | b. $K_4 \text{Fe}(CN)_6 M/I 000$ | 17.5                                      | 13           |      |
|                | c. KCNS M/90                     | 20.4                                      | — I          |      |
|                | d. MgCl <sub>2</sub> M/250       | 56.3                                      |              |      |
|                | b. + d.                          | 33.8                                      | 40           |      |
|                | c. + d.                          | 33.8                                      | 40           |      |
| VI             | a. o                             | 112                                       |              | 6.8  |
|                | b. CaCl <sub>2</sub> M/4 000     | 99                                        | I 2          |      |
|                | c. phosphate M/90                | 90                                        | 20           |      |
|                | b. + c.                          | 41                                        | 63.5         |      |

were not investigated). M/100 Na<sub>2</sub>SO<sub>4</sub> and Na phosphate, M/1000 K<sub>4</sub>Fe(CN)<sub>6</sub> and M/90 KCNS which by themselves show little or no inhibition of aerobic glycolysis, strongly inhibit in presence of M/250 MgCl<sub>2</sub>, which by itself increases the glycolysis. The inhibition by M/4000 Ca in presence of M/100 Na<sub>2</sub>SO<sub>4</sub> or Na phosphate is much stronger than corresponds to the sum of inhibitions of the two kinds of ions (Table IV). This synergy manifests itself also towards the oxidation of glucose as well as towards the original O<sub>2</sub> consumption of the hemolysate. On the other hand no synergy was found between K and Na<sub>2</sub>SO<sub>4</sub> or Na phosphate.

f) Reversibility of the inhibitory effect of Ca against the aerobic glycolysis. That the inhibition of the metabolism in the hemolysate by ions is not due to an irreversible destruction of enzymes is clearly indicated by the fact that the degree of the inhibition does not increase with the time even when the inhibition was not complete. The reversibility of the inhibition was, furthermore, demonstrated directly for Ca in the following way. Two samples of washed red cells were taken. One sample, hemolysate I, was hemolyzed with 1.5 volumes of water containing enough Ca to yield a final concentration of 2 mg % in the hemolysate. The other sample, hemolysate II, was hemolyzed with 1.5 volumes of water. 4 samples of 1 ml each were pipetted from every hemolysate. 0.03 ml of a 2% glucose solution were added to samples of hemolysate I and I sample of hemolysate II (glucose samples) while to the remaining five samples 0.03 ml of water was added (water samples). All samples were left for 2 hours at 25° and then the glucose sample and one water sample of II and one of the glucose samples of I and one water sample were deproteinized (2 hours samples). 0.6 ml of water was now added to the glucose and water samples of I and to the one of the water samples of II while the other water sample of II received 0.6 ml of a glucose solution of 0.1%. The Ca concentration in I was thus reduced from 2 to 1.2 mg %. If the inhibition of the aerobic glycolysis by Ca was reversible then the reduction of the Ca concentration in I should result in a decrease of the inhibition in the following 2 hours. This was in fact the case.

It must be noted that the 4 hour glucose sample of I contained more lactic acid in the second 2 hour period than the corresponding sample of II. This tended to make the inhibition by Ca rather stronger than weaker.

TABLE VI balance of glucose consumption,  $\rm O_2$  uptake and lactic acid formation in the hemolysate. 4 hour experiments at  $25^{\circ}$  ph 7.0

|             |                                   | ptake in γ/<br>hemolysat          |     | Increase<br>of CO <sub>2</sub> | Glucose                  | Lactic         | C<br>Oguptake               | D                          | Ratio         |
|-------------|-----------------------------------|-----------------------------------|-----|--------------------------------|--------------------------|----------------|-----------------------------|----------------------------|---------------|
| Exp.<br>No. | A<br>by hemo-<br>lysate<br>itself | B<br>hemoly-<br>sate +<br>glucose | В-А | produc-<br>tion by<br>glucose  | con-<br>sumed<br>in γ/ml | acid<br>formed | due to glucose in $\mu$ mol | mol<br>glucose<br>oxydized | $\frac{C}{D}$ |
| 33          | 50                                | 75                                | 25  |                                | 255                      | 163            | 0.78                        | 0.51                       | 1.53          |
| 35          | 46                                | 6 r                               | 15  |                                | 250                      | 207            | 0.47                        | 0.24                       | 1.96          |
| 40          | 86                                | 115                               | 29  | 21.5                           | 525                      | 320            | 0.9                         | 1.14                       | 0.79          |

#### DISCUSSION

# 5. Mechanism of the aerobic glycolysis in the hemolysate

On the basis of our experiments we can draw the conclusion that the aerobic metabolism in nucleated erythrocytes consists of several distinct enzymatic systems. If no glucose is added to the hemolysate no significant amounts of preformed hexoses are available for oxidation, but adenosine-5-phosphate, derived from ATP, breaks down and its ribose disappears. This process and oxidation of fat and protein should be responsible for the observed respiration of the hemolysate in absence of glucose. The increase after addition of glucose can be traced again to at least two different reactions, namely, complete oxidation to  $\mathrm{CO}_2$  and oxidation of glucose to a phosphoric ester, whereby one atom of oxygen combines with one mol of glucose. It is probable that the latter reaction consists in the oxidation of glucose to phosphogluconic acid.

The powerful aerobic glycolysis in the hemolysate in presence of glucose can be due to the fact that the oxidation of one molecule of glucose is coupled with the phosphorylation of many molecules of this sugar and the triosephosphate dehydrogenase is much more efficient in the hemolysate than the system oxidizing pyruvate. The excess of the latter is therefore reduced to lactic acid. As the hemolysate contains the enzyme system of the tricarboxylic acid cycle it is reasonable to assume that the oxidation of glucose to CO<sub>2</sub> goes over this cycle. It is known from experiments on other tissue extracts that the oxidation of I mol of glucose in this way can be coupled with the phosphorylation of I8 molecules of glucose to hexose diphosphate. This would explain the fact that the addition of all those acids which increase the turnover of the Krebs cycle, and of Mg which is an activator of the oxidation of pyruvic acid, considerably increases the aerobic glycolysis.

The inability of the hemolysate to glycolyse anaerobically can be explained easily. The hemolysis of nucleated erythrocytes is accompanied by an explosive increase in the activity of ATPase. At room temperature practically all of ATP originally present in the cells is dephosphorylated in a few minutes; the glycolysis of one molecule of glucose can maximally resynthesize 2 molecules of ATP. As long, therefore, as the speed of the simple dephosphorylation of ATP exceeds the speed of transphosphorylation with glucose the latter process must stop in anaerobiosis due to the total disappearance of ATP. The efficiency of the oxidative breakdown of glucose as far as synthesis of ATP is concerned makes it possible to keep up under aerobic conditions a certain minimum concentration of ATP necessary for the phosphorylation of glucose. This amount, however, is very small, even under aerobic conditions, and not detectable by the usual colorimetric procedures of determination.

# Point of attack of ions

The realization of this multitude of enzymatic processes involved in the aerobic metabolism is important for the consideration of the possible mechanism of the inhibitory effects of ions on this metabolism. It appears significant that all ions, cations as well as anions, are able to suppress not one but many of the enzyme reactions constituting the oxidative metabolism. On the other hand, the degree of inhibition is different for different enzyme reactions or systems of reactions. The aerobic glycolysis is in general more strongly inhibited than the oxidation of glucose, which in turn suffers more than the  $O_2$  consumption without glucose. The oxidation of succinate and  $\alpha$ -ketoglutarate

are least affected. It is very significant that this sequence in the susceptibility to inhibitory effects is the same for all kinds of ions and the reactions affected are of very different types. The oxidation of glucose, for example, is as was shown due to two completely different reactions. It appears most improbable that so many and so different reactions should be influenced in the same way by all the ions. We have rather to assume that the ions exert their influence on a substrate the activity of which is again correlated is some way with the activities of all the enzymes of the oxidative system. Such a substrate for example is the cytochrome system, which serves as H<sub>2</sub> carrier to the oxidation of the preformed substrates of the hemolysate as well as that of added glucose. It seems impossible, however, to consider the cytochrome system as the point of attack in the ionic inhibition, because of the great differences between various enzymes in their sensitivity towards the ions. M/1000 of Ca almost completely inhibits the oxidation of glucose, but the inhibition of the basic respiration of the hemolysate is not complete even at M/200. M/500 HCN, on the other side, inhibits both to the same extent. All this suggests that the inhibitory action of ions is directed against one single substrate which in changing its physicochemical properties influences in its turn all the enzymes of the oxidative system. The enzymes are in fact not in solution inside the cell, but are attached to insoluble particles, the mitochondria. These contain, apart from proteins, considerable amounts of lipids and ribosenucleic acids. In these subcellular structural and functional units the enzyme proteins are probably attached to a stroma consisting of lipo- and nucleoproteins and may be surrounded by a surface membrane. One way to explain the effects of ions on the oxidative processes would therefore be to assume a decrease in the permeability of such a surface membrane under their influence. The fact that the aerobic glycolysis coupled with the oxidations is quite generally more strongly inhibited than the oxidative processes themselves is in agreement with this concept. This glycolysis depends on the coupled phosphorylation of a phosphate carrier which transfers the phosphate to glucose. Any decrease in the permeability of the surface membrane will decrease the speed of the penetration not only of the substrate but also of the phosphate carrier and the speed with which it leaves the particle after being phosphorylated. The amounts of the phosphate carrier available for the reaction with glucose must decrease to a much higher degree than the corresponding oxidative process. It could also be that the ions change not the permeability but the physical properties of the hypothetical stroma to which the enzyme proteins are attached. Any change in the water binding capacity or shape of the protein molecules of the stroma would have a considerable influence on the shape and arrangement of the respective enzyme proteins and tend to change their activity.

If we assume that in one way or the other the proteins of mitochondria are the point of attack of inhibiting ions the most probable mechanism of this inhibitions appears to be elimination of local electric fields on the surface of this protein, due to the adsorption of the ions. Thus CNS<sup>-</sup> which forms stable complexes with proteins, inhibits the aerobic glycolysis at low concentrations, whereas Cl<sup>-</sup> is ineffective. This can also explain the characteristic synergy between cations and anions in their effects. Even at the isoelectric point of a protein the charged groups on its surface will exert con-

<sup>\*</sup> More recent experiments on the mechanism of the inhibition of the oxidative enzymes by Ca<sup>++</sup> suggest, that the specific protein in the mitochondria, affected by ions, influences the energy transfer during the enzymereactions of the Krebs cycle rather, than the enzymes themselves or the access of substrates. The results of these new experiments will be reported in a subsequent paper.

siderable forces of repulsion on ions of the same charge and thus counteract their adsorption. This repulsion will obviously be decreased by the simultaneous presence in solution of ions of opposite charge of great surface activity. The adsorption of cations will therefore be facilitated by the presence of easily adsorbable polyvalent anions, and vice versa, and thus a higher degree of elimination of polarized groups on the protein surface may be achieved. This again will affect the water binding capacity and the shape of the respective protein molecule.

This view appears supported by the rather striking analogy between the inhibition of the aerobic metabolism by ions and the effect of certain ions on proteins like myosin, actin, actomyosin and the so called structural proteins of kidney and brain investigated by SZENT-GYÖRGYI and his associates<sup>12</sup>. These proteins adsorb physiological cations (Na, K, Ca, Mg) from solutions of physiological concentrations. Ca is more strongly adsorbed than Mg and this again more strongly than the monovalent cations. This adsorption neutralizes charges of polar groups on the protein surface and changes the affinity to water and in the case of actin the ability to polymerize. A striking analogy to the synergy between ions in our case can be seen in the influence of the cations (K, Ca) on the adsorption of the polyvalent ATP ion by myosin. In this case the anion of ATP does not counteract the effect of K on myosin but enhances it.

The affinity of structural proteins to ions depends upon a certain specific state of the protein surface and is easily suppressed by procedures tending to denature the protein. The adsorption of cations by myosin for example decreases strongly during 24 hour storage at 0°13. This may be the reason why such general inhibitory effects of ions on oxidative enzymes have not yet been observed in tissue homogenates. In this case the subcellular structural units may suffer considerable injury by the mechanical crushing of the tissue. Hemolysis on the other hand appears as a much milder procedure for getting access to a little altered inner parts of the cell.

#### SUMMARY

- 1. The hemolyzed nucleated erythrocytes of the pigeon show considerable  $\rm O_2$  consumption, which is considerably increased by MgCl<sub>2</sub> M/250, glucose and constituents of the tricarboxylic acid cycle and completely inhibited by NaCN M/250.
  - 2. This oxidative metabolism is coupled with a strong aerobic glycolysis.
  - 3. All multivalent cations and anions inhibit the  ${\rm O_2}$  consumption as well as the aerobic glycolysis.
  - 4. CaCl<sub>2</sub>, orthophosphate and ribonucleate inhibit strongly at physiological concentrations.
  - 5. Different oxidative reactions in the hemolysate are inhibited by ions to a different degree.
- 6. These inhibitory effects of ions may be due to disturbances of the local electric fields of proteins which are constituents either of membrane or stroma of subcellular structural units which are carriers of enzymes of the oxidative system of the cell.

#### RÉSUMÉ

- 1. Les nucléo-érythrocytes hémolysés du pigeon montrent une consommation d'oxygène considérable, qui est encore fortement accrue par  $\mathrm{MgCl_2\ M/25o}$ , le glucose et les constituants du cycle des acides tricarboxyliques, mais complètement inhibée par NaCN  $\mathrm{M/25o}$ .
  - 2. Ce métabolisme d'oxydation est couplé avec une forte glycolyse aérobique.
- 3. Tous les cations et anions plurivalents inhibent la consommation d'oxygène aussi bien que la glycolyse aérobique.
- 4. Le CaCl<sub>2</sub>, l'ion orthophosphorique et l'ion ribonucléique sont de forts inhibiteurs aux concentrations physiologiques.
- 5. Différentes réactions d'oxydation, dont l'hémolysat est le siège, sont inhibées par les ions à des degrés différents.
- 6. Ces effects inhibitoires d'ions sont peut-être dus à des perturbations des champs électriques locaux des protéines qui sont des constituants soit de la membrane, soit du tissus conjonctif d'unités structurales subcellulaires, supports d'enzymes du système d'oxydation de la cellule.

#### ZUSAMMENFASSUNG

- 1. Die hämolysierten, kernhaltigen Erythrocyten der Taube zeigen einen bedeutenden  $O_2$ -Verbrauch, welcher durch  $\operatorname{MgCl}_2$   $\operatorname{M}/250$ , Glucose und Bestandteile des Tricarboxylsäure-Zyklus beträchtlich erhöht, durch  $\operatorname{NaCN}$   $\operatorname{M}/250$  dagegen völlig unterbunden wird.
  - 2. Dieser oxydative Metabolismus ist mit starker aerober Glykolyse gekuppelt.
- 3. Alle mehrwertigen Kationen und Anionen hemmen den  $O_2$ -Verbrauch sowohl als die aerobe Glykolyse.
  - 4. CaCl<sub>2</sub>, Orthophosphat und Ribonukleinat hemmen bei physiologischer Konzentration stark.
- 5. Verschiedene oxydative Vorgänge im Hämolysat werden durch Ionen verschieden stark gehemmt.
- 6. Diese hemmenden Wirkungen der Ionen beruhen vielleicht auf Störungen lokaler elektrischer Felder von Proteinen, welche Bestandteile sind von Membran oder Bindegewebe von subcellularen Struktureinheiten, die Träger von Enzymen des oxydativen Systems in der Zelle sind.

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# THE BIOCHEMISTRY OF ABNORMALITIES IN CELL DIVISION

by

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Carbohydrates have been considered for a long time to be the fuel of the tissues of the body, but it is only during the last few years that some of the mechanisms whereby the energy from carbohydrate catabolism is utilized have been revealed. MEYERHOF has done more than any other biochemist to show how carbohydrate metabolism involves phosphorylation and how the phosphorylated products can yield energy for other biological processes. A remarkable property of living machinery is that it can make, repair and maintain its own working parts. Cancer tissue has a high carbohydrate metabolism and a high rate of cell division. The carbohydrate metabolism, partly aerobic and partly anaerobic, yields the energy necessary for cell division and the maintenance of the nuclei which seem to control the processes of cell division. The main constituents of cell nuclei of both normal and cancer cells appear to be proteins and nucleic acids, and the carbohydrate metabolism is possibly merely concerned with production of high energy phosphate bonds which will yield energy in a form available for synthesis of nucleic acids and possibly of proteins. Inhibition of these processes will stop cell division and so inhibit growth. If the inhibition is such that cell division is impeded but not stopped then the incidence of abnormalities such as damaged chromosomes, mutations or cancer might be increased.

Normal cells are not capable of continuous growth. If they continue to receive surplus nourishment after attaining a certain limiting size they divide. If the process of cell division is inhibited, then growth is also inhibited. In the cell division or mitosis in which nuclei and plasmagenes play a dominant rôle there is exact partitioning of the chromosome material between the daughter cells. The occurrence of spontaneous chromosome abnormalities and mutations shows that chromosomes are not absolutely stable. The induction of changes or mutations by physical and chemical agents indicates that the nuclear material is sensitive and vulnerable to conditions of the environment.

Perhaps the most sensitive indication of abnormalities of cell division is the occurrence of mutations, as these are functional manifestations of such abnormalities. If the change of normal cells to cancer cells is a somatic mutation then the fact that an agent is carcinogenic is an indication that it is mutagenic. Actually most of the mutagenic agents which lend themselves to testing have been found to be carcinogenic and many carcinogenic agents have been shown to induce mutations.

Many of the means which will induce cancer and increase the mutation rate of animals will inhibit the growth of animals or of tumours growing in animals. Such inhibition of growth by carcinogenic hydrocarbons was described by Haddow<sup>1</sup>. Inhibition of growth in this way may form the basis for therapy of cancer.

The more complete correlation between the actions we are considering was first References p. 300.

shown with X-rays. Radiotherapy of cancer started (Grubbe²) soon after Röntgen's discovery of X-rays. Seven years later Frieben³ reported that a skin cancer had developed in a man who had been exposed to X-rays. Muller⁴ showed that X-rays increased the incidence of mutations in Drosophila and Painter and Muller⁵ and Koller⁶ found that X-radiation caused visible abnormalities in chromosomes.

All these effects can be produced by certain chemical agents, such as the nitrogen mustards and urethane, which for this reason have been called radiomimetic. The carcinogenic hydrocarbons such as 1:2:5:6-dibenzanthracene are also radiomimetic agents. Table I shows the grouping of the different effects.

TABLE I
REFERENCES TO EFFECTS PRODUCED BY X-RAYS AND BY CHEMICAL
COMPOUNDS WITH RADIOMIMETIC ACTIONS

|                                                  | Treatment of<br>Cancer or<br>Inhibition of<br>growth             | Induction of<br>Cancer                          | Induction<br>of<br>Mutations                 | Chromosome<br>Damage                                          |
|--------------------------------------------------|------------------------------------------------------------------|-------------------------------------------------|----------------------------------------------|---------------------------------------------------------------|
| X-rays                                           | Grubbe <sup>2</sup>                                              | Frieben <sup>3</sup>                            | Muller4                                      | Painter and<br>Muller <sup>5</sup>                            |
| Nitrogen Mustard                                 | Rhoads <sup>7</sup>                                              | Boyland and<br>Horning <sup>8</sup>             | AUERBACH,<br>ROBSON AND<br>CARR <sup>9</sup> | Boyland, Clegg<br>Koller, Rhoden<br>and Warwick <sup>10</sup> |
| Urethane                                         | Paterson,<br>ApThomas,<br>Haddow, and<br>Watkinson <sup>11</sup> | NETTLESHIP AND<br>HENSHAW <sup>12</sup>         | OEHLKERS <sup>13</sup>                       | BOYLAND AND<br>KOLLER <sup>14</sup>                           |
| 1:2:5:6-Dibenz-<br>anthracene                    | HADDOW, SCOTT,<br>AND SCOTT <sup>15</sup>                        | Kennaway <sup>16</sup>                          | Carr <sup>17</sup>                           | Koller <sup>18</sup>                                          |
| Methylcholanthrene                               | Haddow, Scott,<br>and Scott <sup>15</sup>                        | Cook and<br>Haslewood <sup>19</sup>             | Strong <sup>20</sup>                         |                                                               |
| N:N-di (2-chloro-<br>ethyl)-2-naph-<br>thylamine | Haddow, Kon,<br>and Ross <sup>21</sup>                           | HADDOW,<br>HORNING, AND<br>KOLLER <sup>22</sup> | _                                            | Koller <sup>18</sup>                                          |
| 4-Dimethylamino-<br>stilbene                     | Haddow, Harris,<br>Kon, and Roe <sup>23</sup>                    | Haddow, Harris,<br>Kon, and Roe <sup>23</sup>   |                                              | Koller <sup>18</sup>                                          |

Another effect which many of these agents produce is the bleaching or greying of hair. This was described in mice exposed to X-rays by Hance and Murphy<sup>24</sup>. A similar effect occurs with nitrogen mustard derivatives either aliphatic (Boyland *et al*<sup>10</sup>) or aromatic (Haddow *et al*.<sup>22</sup>). This greying of hair is a permanent effect, remaining with the mouse for the remainder of its life. It may be perhaps considered as a somatic mutation and in this respect is analogous to an induced tumour. The change from coloured to white hair which is induced is unlikely to be due to selective survival of more resistant white hair follicles as the skin of the black (C57) or agouti (CBA) mice used do not appear to contain white hairs. This change of colour in a part of the body is a discontinuous variation in properties like the change of normal into cancer cells. Both changes are brought about by the same agents which also induce germinal mutations.

These agents also cause visible damage to chromosomes and it is probable that the inherited variations are due to change of plasmagenes or to chromosome damage which might not have been visible if the affected cell had been examined. The dose of mutagenic agent which is required to produce visible abnormalities will cause death in many of the treated cells and the new forms arise in the cells which have received a sublethal dose.

The tumours which arise as the result of treatment of cells with a mutagenic agent are possibly derived from a normal host cell which has produced daughter cells differing from the parent cell because of some accidental error or abnormality of cell division. When the total number of cell divisions in the whole mammalian body is taken into account these abnormalities are very infrequent. The chance of their occurrence seems to be made much more probable by the presence of a carcinogenic or mutagenic agent.

If the changes brought about by carcinogenic agents are random variations of the original cells as suggested it is perhaps surprising that different tumours are so similar in their morphology and biochemistry. Each tumour has its own specific characters but the differences between tumours induced by carcinogenic agents are relatively small. Different tumours resemble each other more closely than they resemble the tissue of their origin. Thus tumours have less of the specific functions of the cell from which the tumour arose and tumours have the property of producing lactic acid aerobically. Of the mutations which occur in somatic cells probably many are unable to survive; many will die normally and others will be unable to withstand the attacks of defence processes of the host. Of the numerous mutations which occur only those which produce cells able to survive, grow, and induce the host to provide a blood supply, will become detectable cancers, and for these biological processes specific characters of function and morphology may be required. As the changes are induced by substances which damage the chromosome material (either directly or indirectly) and probably the genes, the changesare probably the result of loss or inactivation of genes, as it seems unlikely that a toxic agent should add something to the nuclear material. Such changes would be analogous to the mutations induced in Neurospora which result in the loss of ability to carry out some specific chemical process.

The biochemical mechanism which operates when radiations, nitrogen mustards or carcinogenic hydrocarbons induce mutations or cancer, is still obscure. The nitrogen mustards or chloroethylamines are chemically reactive and combine with many tissue constituents and inactivate many enzymes, but particularly the phosphokinases and the pyruvic oxidase enzyme system. In order to produce the chromosome damage and inhibition of the growth of tumours in animals the aliphatic chlorethylamines must have two chloroethyl groups (Boyland et al. 10) and the necessity of two reactive or polar groups for chemotherapeutic action against cancer was suggested earlier (BOYLAND<sup>25</sup>). GOLDACRE, LOVELESS, AND ROSS<sup>26</sup> have suggested that the two active groups join chromosome parts by cross linkage of protein or other constituents. As a result of these additional cross linkages the division of chromosomes is hindered and breakages and damage to the chromosomes occurs. This theory would not account for the action of urethane (which seems to have no chemically reactive groups) and it is difficult to see how arsenicals such as sodium arsenite could act in this way. Sulphydryl compounds are the only known tissue constituents with which arsenite is known to react. As there is very little cysteine or other sulphydryl compound in chromosomes (Davidson and Lawrie<sup>27</sup>) combination of chromosome chains by union of sulphydryl groups through an arsenic atom is unlikely to occur. It also seems improbable that X-rays would cause stable cross linkages between chromosome parts to be formed. The current theory of the action of radiations on cells is that they oxidize sulphydryl groups through the production of peroxide or other oxidizing agent within the cells. They could therefore unite peptide chains by conversion of sulphydryl groups to the disulphide forms. The low concentration of cysteine in the chromosomes which was suggested as a difficulty in the theory as applied to the action of arsenicals would also apply to X-rays. A linkage through arsenic might, however, be more stable than a disulphide link which would probably be reduced in processes of cell metabolism. This hypothesis of cross linkage within chromosomes being the cause of abnormalities may be of value in investigating the action of drugs on tumour cells, but it is possibly of no more value than the knowledge that in the chloroethylamine series and other compounds two active groups are required for the biological actions considered.

The hypothesis which the author put forward (BOYLAND<sup>28</sup>) postulates that the effects of these substances are due to inhibition of enzymes, particularly the phosphokinases or enzymes involving oxidative phosphorylations necessary for production and metabolism of the nucleic acid required for the maintenance of normal chromosomes and genes. Since then Barron, Dickman, and Singer<sup>29</sup> have shown that phosphoglyceraldehyde dehydrogenase is particularly sensitive to the action of X-rays, and Meyerhof and Wilson<sup>30</sup> have described the inhibition of hexokinase and phosphohexokinase with phenyl urethane.

Investigations carried out during the war showed that two enzyme systems were particularly sensitive to the poisoning action of vesicants. Of the phosphokinases, hexokinase was first shown by DIXON AND NEEDHAM31 to be inhibited by low concentrations of mustard gas and nitrogen mustard. Later Cori and his co-workers32 found that phosphokinases in general are inhibited by vesicants. Peters, Sinclair, and Thompson<sup>33</sup> found that the arsenical vesicant, lewisite and other vesicants inhibit the pyruvic oxidase system. The known phosphate transferring enzymes are concerned with the building up of energy rich phosphate bonds in phosphoric anhydrides and acylphosphates. Enzymes of this type must be concerned in the biosynthesis of the nucleotides and nucleic acids. Although we know very little of the specific phosphokinases involved in nucleic acid synthesis, the fact that all known phosphokinases are easily inhibited by sulphur mustard and nitrogen mustards would suggest that nucleic acid synthesis should be inhibited by these substances. The synthesis of proteins may also involve phosphorylation of the terminal carboxyl group of a peptide chain and reaction of the resulting acyl phosphate with a fresh amino acid molecule to give a new peptide link and liberate phosphate. A model for this reaction is the formation of glutamine from phosphoryl glutamic acid and ammonia (Speck34, Elliot35). The enzymes concerned with nucleic acid and protein synthesis need investigation and for this the mitotic poisons may be useful tools.

The substances which induce mitotic abnormalities differ greatly in their apparent chemical reactivity. The aliphatic nitrogen mustards are very reactive substances, the aromatic chloroethylamines react slowly, but the aromatic carcinogenic hydrocarbons are rather inert. The French theoretical chemists Daudel, Pullman and their associates (Daudel<sup>36</sup>) have shown that the carcinogenic hydrocarbons have regions, known as the K regions, in which there is high electron density, which in the majority of the carcinogenic hydrocarbons includes an activated phenanthrene double bond. The activation is enhanced by substituents such as benzene rings or methyl groups (which repel elec-

trons) in such positions that they increase the electron density of the phenanthrene double bond. This double bond in the more potent carcinogenic hydrocarbons such as 9:10-dimethylbenzanthracene has a chemical reactivity for some addition reactions approaching that of an aliphatic ethylenic bond and even greater than that of the ethylene bond of some stilbenes. This theory which is now substantiated by experimental evidence, suggests that the more active carcinogens in any particular series of aromatic compounds are those which are on the whole the more chemically reactive.

Phenanthrene itself reacts readily with osmic acid (CRIEGEE, MARCHAND, AND WANNOWIUS<sup>37</sup>) and the carcinogenic hydrocarbons react even more rapidly (BADGER<sup>28</sup>). Osmic acid adds on to the double bond of the K region to form an adduct, which can be easily hydrolysed to give *cis*-dihydroxydihydro-derivatives.

Perbenzoic acid is another reagent which appears to react with carcinogenic hydrocarbons at rates varying with the carcinogenic activity. This reagent was shown to react with 20-methylcholanthrene and 3.4-benzpyrene more rapidly than with anthracene and phenanthrene (Eckhardt³) before the theory of the K region of carcinogens had been developed. In looking for a means of measuring the relative reactivity of the K region, the reaction of perbenzoic acid with a series of carcinogens has been determined. Some of the data obtained are shown in Table II. The figures show that the carcinogenic hydrocarbons react at about the same rate as the carcinogenic aminostilbenes. This suggests that the bond of the K region of the hydrocarbons is as reactive as the ethylenic bond of the stilbene molecule and as the azo group of the carcinogenic dimethylaminoazobenzene.

The fact that dimethylaminoazobenzene dosed to animals in which it induces hepatoma is found in a combined form in the protein of the liver (MILLER AND MILLER<sup>40</sup>),

TABLE II

REACTION OF CARCINOGENS AND RELATED SUBSTANCES WITH PERBENZOIC ACID

M/50 solutions of substances dissolved in carbon tetrachloride with M/50 perbenzoic acid at 25° C.

The remaining perbenzoic acid was estimated iodometrically and the results are expressed as millimols of perbenzoic acid used per mol substrate.

| Common d                          |     | Carcinogeni |     |     |          |
|-----------------------------------|-----|-------------|-----|-----|----------|
| Compound                          | 3   | 24          | 48  | 72  | activity |
| ): 10-Anthraquinone               | 5   | o           | 0   | 5   |          |
| Naphthalene                       | 0   | 0           | 20  | 10  |          |
| Phenanthrene                      | 5   | 15          | 20  | 25  |          |
| ; 10-Phenanthraquinone            | 0   | 0           | 25  | 35  |          |
| Anthracene                        | 5   | 35          | 60  | 80  |          |
| :2-Benzanthracene                 | o   | 25          | 70  | 95  | -        |
| :2:5:6-Dibenzanthracene           | O   | 15          | 3.5 | 95  | +        |
| -Methyl-1:2-benzanthracene        | 0   | 40          | 40  | 110 | +        |
| -Aminostilbene                    | 32  | 85          | 120 | 140 | +        |
| -Acetylaminofluorene              | 0   | 40          | 100 | 160 | +        |
| :4-Benzpyrene                     | 5   | 90          | 130 | 202 | +        |
| :4-Benzphenanthrene               | 45  | 90          | 145 | 220 | +        |
| tilbene                           | 5   | 20          | 110 | 295 | ?        |
| :4:5:6-Dibenzcarbazole            | 95  | 215         | 265 | 322 | +        |
| o-Methylcholanthrene              | 105 | 275         | 340 | 405 | +        |
| '-Methyl-4-dimethyl-aminostilbene | 215 | 405         | 465 | 535 | +        |
| '-Chlor-4-dimethyl-aminostilbene  | 175 | 390         | 500 | 590 | +        |
| Dimethylaminoazobenzene           | 455 | 590         | 615 | _   | +        |

shows that a carcinogen can react with tissue protein. As the hydrocarbons react with perbenzoic acid almost as rapidly as dimethylaminobenzene and the azo group of the latter compound is expected on theoretical grounds to have an electron density of the same order as the carcinogenic hydrocarbons, the carcinogenic hydrocarbons might also be expected to combine with some tissue protein in a similar way.

Although the French theoretical chemists have concentrated on the K region of a particular carcinogenic hydrocarbon it is perhaps worth noticing that these substances have two active regions. Many carcinogens such as 1:2:5:6-dibenzanthracene and 3:4benzphenanthrene contain two active phenanthrene double bonds or K regions. In those carcinogenic hydrocarbons with only a single K region the groups which activate that region may also increase the activity of a second part of the molecule. Thus, in the potent carcinogen 9:10-dimethyl-1:2-benzanthracene, the two methyl groups not only make the 3:4 bond more active than in the unsubstituted 1:2-benzanthracene but also increase the chemical reactivity of the 9:10 or meso positions. Such meso substituted anthracene derivatives are extremely susceptible to many chemical reactions, such as photo-oxidation. The metabolism of carcinogens also shows that another region of the molecule (the benzene ring adjoining the K region) is liable to attack in vivo. Although it is quite clear that carcinogenic hydrocarbons must have one centre of high chemical reactivity, they also have a second active centre, either a second phenanthrene double bond, active meso positions, or an amino group as in the aminostilbenes or the aminoazobenzene derivatives.

The reactivity of hydrocarbons is also shown by metabolism experiments with noncarcinogenic hydrocarbons such as naphthalene (Booth and Boyland<sup>41</sup>); (Young<sup>42</sup>) and anthracene (BOYLAND AND LEVI43) as well as with the carcinogenic hydrocarbon 3:4-benzpyrene (Weigert and Mottram<sup>44</sup>). These hydrocarbons undergo the reaction of perhydroxylation involving the addition of the elements of hydrogen peroxide with formation of dihydroxydihydro derivatives or diols. In the case of the non-carcinogenic hydrocarbons the addition of the hydroxyl groups occurs at the centres with highest electron density. But in the carcinogenic hydrocarbons which have been examined the oxidation occurs in positions in a ring adjacent to the K region — not in the reactive K region itself. This may be because the more reactive carcinogens combine with some tissue constituent through the double bond so that only regions of secondary activity are available for the oxidative process. The investigation of 3:4-benzpyrene metabolism showed that the dihydroxydihydro-benzpyrene formed by metabolism in isolated skin was combined to some tissue constituent. The combination, however, could be destroyed by treatment with wet acetone. Studies with 1:2:5:6-dibenzanthracene containing radioactive carbon (Heidelberger and Jones 45) have shown that a small part of the carcinogen remains in animals for many months after injection. Thus there are several indications, that the carcinogenic hydrocarbons can react with some, as yet unidentified, tissue constituents.

Although these hydrocarbons have some of the biological effects of nitrogen mustards they do not appear to inhibit the hexokinase of tumours; the anaerobic glycolysis and respiration of tumours is the same whether they are growing normally or are inhibited by 1:2:5:6-dibenzanthracene (BOYLAND AND BOYLAND<sup>46</sup>). On the other hand inhibition of tumour growth by nitrogen mustard is accompanied by a decrease in the anaerobic glycolysis of the tissue (BOYLAND et al.<sup>10</sup>). This inhibition of tumour growth by carcinogens, such as 4-dimethylaminostilbene or 1:2:5:6-dibenzanthracene, is only

seen if the treated animals are maintained on a low protein diet (Elson and Haddow<sup>47</sup>). This finding indicates that the inhibition of growth is probably due to interference with protein metabolism which can be overcome if the protein intake of the host is sufficiently high. As 1:2:5:6-dibenzanthracene causes abnormalities of chromosomes these experiments suggest that chromosomes require an adequate supply of amino-acids for their proper maintenance.

The rates of diffusion and reaction are probably important characteristics of the nuclear poisons which have been discussed. The compounds must, presumably, react in or near the nucleus to produce their effects. For this they must diffuse through the cell to the nucleus more rapidly than they react with the constituents of the tissue through which they are passing, unless they have a specific affinity for the particular constituents concerned with nuclear behaviour. The aliphatic nitrogen mustards react very rapidly in the body, having a life of only a few minutes, but they do not react instantaneously with any reagent and diffuse rapidly so that some unchanged molecules may reach the nucleus.

The evidence put forward supports the theory that chemical carcinogenic and therapeutic agents for cancer combine with tissue constituents and that physical agents cause some chemical change in chromosome constituents. Goldacre, Loveless, and Ross<sup>26</sup> suggest that it is the chromosomes themselves which are affected while the author considers that the effects are due to inhibition of enzymes concerned in metabolic processes involved in maintenance and functioning of the chromosomes.

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#### SUMMARY

- r. The association of the effects of chromosome damage, induction of mutations and induction of cancer with a number of agents is discussed.
- 2. Examination of the reaction of a series of carcinogenic compounds with perbenzoic acid shows that carcinogenic hydrocarbons react more rapidly than simpler non-carcinogenic hydrocarbons and at about the same rate as nitrogenous aromatic carcinogens.
- 3. The suggestion that the nitrogen mustards and possibly other carcinogens produce their effects by inhibition of enzymes necessary for normal functioning of cell nuclei is considered.

#### RÉSUMÉ

- 1. La relation entre les lésions des chromosomes, l'induction de mutations et l'induction du cancer par un nombre d'agents est discutée.
- 2. L'examen de la réaction d'une série de composés cancérigènes avec l'acide perbenzoïque démontre que les hydrocarbures cancérigènes réagissent plus rapidement que les hydrocarbures non-cancérigènes et à la même vitesse à peu près que les substances cancérigènes azotées aromatiques.
- 3. La suggestion que les moutardes azotées et peut-être d'autres substances cancérigènes produisent leurs effets en inhibant les enzymes nécessaires pour le fonctionnement normal du noyau cellulaire est considérée.

#### ZUSAMMENFASSUNG

- r. Das Verhältnis zwischen Chromosomenverletzung, Hervorrufen von Mutationen und Krebsbildung durch verschiedene Agentien wird diskutiert.
- 2. Die Untersuchung der Reaktionen einer Reihe von cancerogenen Verbindungen mit Per-References p. 300.

benzoesäure zeigt, dass die cancerogenen Kohlenwasserstoffe schneller reagieren als einfachere, nicht cancerogene Kohlenwasserstoffe und ungefähr ebenso schnell wie stickstoffhaltige aromatische Krebsstoffe.

3. Der Verfasser schlägt vor, dass die Chloräthylamine und möglicherweise auch andere cancerogene Substanzen ihre Wirkung durch Hemmung der für die normale Funktion der Zellkerne notwendigen Enzyme ausüben könnten.

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# LIPASE-CATALYSED CONDENSATION OF FATTY ACIDS WITH HYDROXYLAMINE

by

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Some time ago we reported preliminarily on two different types of enzymatic reactions leading to a condensation with hydroxylamine<sup>1</sup>. Acetate when incubated with adenosine triphosphate and hydroxylamine was found to yield acet-hydroxamic acid in fresh pigeon liver extracts. This reaction is specific for acetate, depends strictly on ATP, and occurs only in fresh liver extract of the pigeon but not of rat, rabbit or hog. The reaction is lost with aging but is regenerated on addition of coenzyme A and thus belongs in a class with the coenzyme A dependent acetyl transfer reaction. The characteristics of this type of hydroxamic acid acid formation will be reported on elsewhere in more detail.

The second reaction was of an entirely different type. It occurred only with higher concentrations of hydroxylamine and was fully independent of ATP. In the meantime we studied this reaction extensively and are reporting here the results obtained. It is found to occur only weakly with acetate but increasingly with the lengthening of the fatty acid chain, up to an optimum at octanoate. It is present in comparable strength in all liver extracts studied so far. It does not diminish appreciably on aging or dialysis. In contrast to the acetate reaction with ATP, it was strongly inhibited by fluoride. This and other observations eventually led to the conclusion that we were dealing here with a lipase-catalysed condensation of the fatty acid carboxyl with hydroxylamine.

#### METHODS AND ENZYME PREPARATIONS

Hydroxamic Acid determination. — The previously described method² was designed for a determination of acyl phosphate formed during enzymatic incubation. Hydroxylamine was added at the end of incubation to react non-enzymatically with pre-formed acyl phosphate at a  $p_H$  of slightly above 6. Subsequently, after deproteinization with trichloracetic acid, the color was developed with acid ferric chloride. In contrast to this earlier set-up, the hydroxylamine now is part of the reaction system and is present during incubation; the method is modified to determine the enzymatically formed hydroxamic acid. The experiment is generally terminated by addition of a mixture of trichloracetic acid, hydrochloric acid and additional hydroxylamine. Finally ferric chloride is added. The addition of hydroxylamine serves only to stabilize the color but does not participate in primary

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<sup>\*</sup> I am happy for the opportunity to express with this contribution my gratitude and increasingly realized indebtedness to Professor Otto Meyerhof and his laboratory for what I imbibed there during my apprenticeship from 1927–1930.

condensation. As previously described, the precipitate is eventually removed by filtration or centrifugation and the color determined in the supernatant.

Determination in 50% alcoholic solution.— When it appeared desirable to follow the hydroxamic acid formation with fatty acids of increasing chain length, it was observed that these hydroxamic acids became increasingly insoluble in water and on removing the protein precipitate, considerable

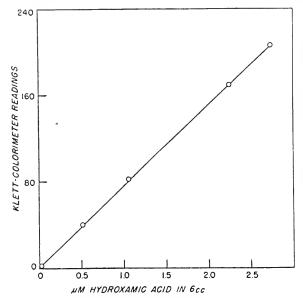


Fig. 1. Standard curve for hydroxamic acid determination in 50% ethanol. Lithium acetyl phosphate was used.

amounts were lost. It was found, however, that these longer chain hydroxamic acids are easily soluble in 50% ethyl alcohol. Therefore in the experiments dealing with higher fatty acids, a revised procedure was used where, after incubation, the medium was brought to a concentration of approximately 50% in ethyl alcohol.

Procedure of Hydroxamic Acid Determination in Alcoholic Solution. — To 0.5 ml of enzyme-substrate-hydroxylamine mixture, 3 ml of 95% ethanol are added and well mixed. Then

1. 1.5 ml are added of a mixture of equal volumes of 28% hydroxylamine-HCl, 3.5 normal NaOH and a hydrochloric acid, obtained by dilution of concentrated HCl with 2 volumes of water,

2. 0.5 ml of 24% trichloracetic acid and finally,

3. 0.5 ml of 10% ferric chloride in 0.2 normal HCl are added. The precipitate is filtered or centrifuged off and the color measured in the supernatant. The main change of procedure is in the use of more highly concentrated solutions in order to keep the volume down and give space for the addition of ethanol.

Since the appearance of our original method, an interesting application of the hydroxamic acid-iron colour for colori-

metry of fatty acid esters appeared<sup>3</sup>. Esters were found to react quantitatively with hydroxylamine in strongly alkaline solution and this reaction is used by Hill<sup>3</sup> for a determination of fatty acid esters. An extensive and very instructive discussion of the reaction between hydroxylamine and carboxyl derivatives may be found in the spot test analysis of Fritz Feigl<sup>4</sup>.

#### ENZYME PREPARATIONS

Pigeon and rat liver homogenate were prepared as described previously<sup>5</sup> using 3 to 4 volumes of 1% potassium chloride and 0.02 M sodium bicarbonate solution.

Hog liver fractionation.— In this fractionation we followed roughly the procedure elaborated for the purification of liver lipase by King and his collaborators<sup>6–9</sup>. Fresh hog liver was obtained from the slaughterhouse and 100 grams were homogenized in a Waring blender with 200 ml of 0.1 molar disodium hydrogen phosphate. The homogenate was frozen overnight and then centrifuged for half an hour after thawing.

Fraction L-1, obtained by removal of inactive protein by acidification. — 75 ml of the extract were further diluted with 2 volumes of 0.1 molar secondary phosphate and recentrifuged. To the supernatant 75 ml of water were added and the mixture was now acidified with 11.5 ml of normal acetic wherewith the p<sub>H</sub> was brought to 4.8. A voluminous precipitate formed and was centrifuged off and discarded. 127 ml of strongly reddish, almost clear supernatant were collected. The extract was neutralized with 5 ml of normal ammonia to p<sub>H</sub> 6.8. 10 ml were taken for analysis.

Fraction L-2, obtained by removal of inactive protein by half saturation with ammonium sulphate.—
122 ml of fraction L-1 were mixed with an equal volume of saturated ammonium sulphate solution.
The mixture was shortly warmed to 30° and filtered. The filtrate was dialysed against distilled water.

Fraction L-3, 50% ammonium sulphate precipitate.— The precipitate on the filter was squeezed between filter paper layers and dried as far as possible. The precipitate was dissolved in about 10 ml of water and dialysed in cellophane against 4 liter of distilled water overnight in the cold room. Next morning the globulin precipitate formed on dialysis was centrifuged and once washed with water.

The precipitate was dissolved with Krebs-Ringer containing o.or molar ammonium hydroxide in a 10 ml of Krebs-Ringer containing o.or ammonium hydroxide. Most of it went into solution and a little undissolved was discarded. This fraction L-3 was practically inactive.

Fraction L-4 obtained by full saturation with ammonium sulphate. — This is the most active fraction. To the half saturated ammonium sulphate solution (L-2) 37 grams per 100 ml of solid ammonium sulphate were added. The total volume of 250 ml obtained. This was warmed to  $30-35^{\circ}$  and filtered overnight in the cold room. The almost colourless filtrate was discarded. The precipitate was dissolved in 15 ml water; it dissolved very completely to a dark red fluid. It was dialysed against distilled water with agitation at room temperature for  $3\frac{1}{2}$  hours. The volume increased to 32 ml and very little precipitate was formed, which we centrifuged off and discarded. This is fraction L-4.

#### Pancreas Lipase

Pancreatine Parke-Davis as obtainable on the market was used. Some fractionation of this product is described later on in the text.

#### RESULTS

In the first two tables, the lipase-catalysed hydroxamic acid formation is compared with the acetate + ATP reaction. In Table I, the inactivity of ATP with octanoate is contrasted with its action on acet-hydroxamic acid formation. It appears that the optimum concentration of hydroxylamine with ATP and acetate is 0.02 molar and that at 0.05 molar already an inhibition is observed. Table II shows the effect of increased concentrations of hydroxylamine on the condensation with octanoate. The strong dependence of this reaction on the high concentration of hydroxylamine will be noted as well as its independence on the presence of ATP. In the further study generally an 0.4–0.6 molar concentration of hydroxylamine was used.

TABLE I HYDROXAMIC ACID FORMATION WITH ACETATE + ATP AT VARIOUS CONCENTRATIONS OF HYDROXYLAMINE All tubes contained 0.5 ml of 10% fresh acetone pigeon liver extract in a total volume of 1.1 ml,  $p_{\rm H}$  7.3, temperature 37°, 60 minutes incubation.

| Octanoate<br>M | Acetate<br>M | $_{\rm M}^{\rm ATP}$ | Hydroxylamine<br>M | Hydroxamic Acid Formed $\mu\mathrm{M}$ |
|----------------|--------------|----------------------|--------------------|----------------------------------------|
|                |              |                      |                    |                                        |
|                | 0.01         | 0.01                 | 0.05               | 0.48                                   |
|                | 0.01         | 0.01                 | 0.02               | 0.48<br>1.08                           |
|                | 0.01         | 0.01                 | 0.01               | 0.78                                   |
| 10.0           |              | 0.01                 | 0.02               | 0.02                                   |
|                |              |                      |                    |                                        |

#### TABLE H

HYDROXAMIC ACID FORMATION FROM OCTANOATE AT HIGHER CONCENTRATION OF HYDROXYLAMINE Each tube contained 0.5 ml rat liver homogenate (1:3 in 1 % KCl, frozen for 4 days) in a total volume of 1.4 ml, adjusted to pH 7.3,  $37^{\circ}$ , 60 minute incubation in air.

| 001 | 0.43 | 1.99                          |
|-----|------|-------------------------------|
| -   | 0.43 | 1.90; 2.1*                    |
| 100 | 0.14 |                               |
| -   | 0.14 | 0. <b>54</b><br>0. <b>4</b> 8 |
|     | 001  | - 0.43                        |

Parallel experiment in a Warburg vessel with nitrogen in the gas phase.

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Table III shows the  $p_H$  optimum of the lipase reaction to be at 7.2. The measurements at the more acid range, however, do not give a true impression of the  $p_H$  dependence. A decrease of activity here is partly caused by the higher concentrations of free fatty acid which is rather strongly inhibitory<sup>8</sup>.

TABLE III  ${\tt THE~PH~OPTIMUM~OF~HYDROXAMIC~ACID~FORMATION~WITH~PORK~LIVER~EXTRACT }$ 

Each tube contained 0.25 ml liver extract, 0.1 ml of 0.1 M octanoate, and 0.15 ml of 2 M hydroxylamine hydrochloride-NaOH buffer, 60 minute incubated at 37°. The buffer was prepared by neutralizing a 4 M hydroxylamine HCl solution with increasing amounts of NaOH and adjusting the volume with water

| Hydroxylamine HCl<br>NaOH | РН         | Hydroxamic Acid Formed<br>μΜ |
|---------------------------|------------|------------------------------|
| 2:0.5                     | 5.9        | 1.39                         |
| 2:1                       | 5.9<br>6.4 | 2.4                          |
| 2:1.5                     | 7.2        | 2.99                         |
| 2:1.75                    | 7.5        | 2.76                         |
| 2:1.95                    | 8          | 1.42                         |

In Table IV, the activity of some lipase inhibitors is recorded. Like lipase the hydroxamic acid reaction is strongly inhibited by fluoride<sup>10</sup> and hexyl resorcinol<sup>7</sup>. The action of benzoate is of some interest. An inhibitory effect of benzoate on the oxidation of butyric but none or less of octanoic acid was observed by Quastel and his collaborators<sup>11</sup>. The hydroxamic acid reaction follows the same pattern of decreased inhibition with increasing chain length of the fatty acids. The inhibition of hydrolytic lipase action of this liver extract was checked manometrically with tributyrin in bicarbonate solution. It was found to a similar extent to be affected by fluoride and hexyl resorcinol; but benzoate showed only a small inhibition of about 10%.

TABLE IV ACTION OF LIPASE INHIBITORS ON HYDROXAMIC ACID FORMATION WITH HOG LIVER EXTRACTS

| Inhibitor           | Concentration | Substrate  | % Inhibition |
|---------------------|---------------|------------|--------------|
| Sodium fluoride     | 0.05 M        | octanoate  | 71           |
|                     | 0.01 M        | octanoate  | 51           |
|                     | 0.003 M       | octanoate  | 35           |
| Sodium benzoate     | 0.01 M        | propionate | 87           |
|                     | 0.01 M        | butyrate   | 83 '         |
|                     | o.oi M        | hexanoate  | 20           |
|                     | 0.01 M        | octanoate  | 15           |
| Hexylresorcinol 0.2 | 25%           | octanoate  | 40           |

In Table V, the lipase action and hydroxamic acid formation are compared with the various fractions, obtained as described above from hog liver extract. The parallel is rather striking. It may be noted that the absolute activity expressed in  $\mu M$  turnover

is considerably smaller in the case of hydroxamic acid formation. The dependence of lipatic hydroxamic acid condensation on higher concentrations of hydroxylamine suggested a near equilibrium situation. Therefore, the influence of the concentration of

| TABLE V                                                |  |  |  |  |  |
|--------------------------------------------------------|--|--|--|--|--|
| COMPARISON OF HYDROXAMIC ACID FORMATION AND TRIBUTYRIN |  |  |  |  |  |
| HYDROLYSIS WITH VARIOUS HOG LIVER FRACTIONS            |  |  |  |  |  |

| Hog Liver Fraction | Hydroxamic Acid<br>μM/6ο' | Tributyrin Split $\mu \text{M}/5'$ |  |
|--------------------|---------------------------|------------------------------------|--|
| Lı                 | 0.73                      | 1.67                               |  |
| L 2                | 0.75                      | 1.6                                |  |
| L <sub>3</sub>     | 0.01                      | 0.03                               |  |
| $L_4$              | 1.2                       | 3.25                               |  |
|                    |                           |                                    |  |

For the hydroxamic acid determination 0.1 of the original fraction was used in a total volume of 0.5 ml, hydroxylamine 0.6 M, and octanoate 0.02 M, and incubated for 60 minutes at 37°. Tributyrin hydrolysis was measured manometrically with the manometer containing the fraction in appropriate dilutions, L1:1/12; L2:1/12; L3:none; L4:1/20. The vessels contained 0.1 ml of the diluted fraction, 0.6 ml of 0.1 M Na bicarbonate and 0.05 tributyrin was dipped in from the side arm. The gas room contained 5% CO<sub>2</sub> in N<sub>2</sub>. To make the two series comparable the values recorded in the table for the manometric experiment were obtained by multiplication with the respective dilution factors.

the other reaction partner, the carboxyl ion, was likewise tested. In Fig. 2, two concentration levels, 0.02 and 0.2 molar are compared. The expected increase with carboxylate concentration is most evident at intermediate chain lengths. With longer chain lengths the often observed inhibition by free long-chain fatty acid overlaps. This also explains the change of the chain length optimum toward shorter chains at higher concentration, due to increasing hydrolysis of the salt at higher concentration levels. It is of special interest that the acetate ion starts to show appreciable activity at the 0.2 molar level.

In Fig. 3 the time curve of the reaction is traced. It appears that, with the reactants present in excess, the condensation occurs practically proportionally with time, indicating, as would be expected, an enzymatic reaction of the zero order.

Although in the experiment with carboxylate ion an intermediate formation of an ester was seemingly excluded, it appeared nevertheless of interest to explore the possibility of rapid enzymatic conversion of ester into hydroxamate. For this purpose, the en-

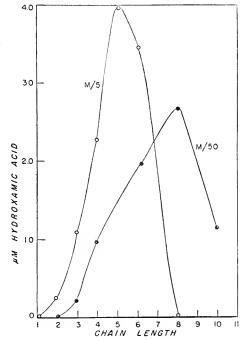


Fig. 2. Comparison of hydroxamic acid formation with 0.02 and 0.2 M octanoate, 0.6M hydroxylamine, and 0.1 ml enzyme solution in 0.5 ml total volume, 60 minutes incubabation at 37°.

zyme was incubated with equivalent amounts of tributyrin and butyrate. As shown

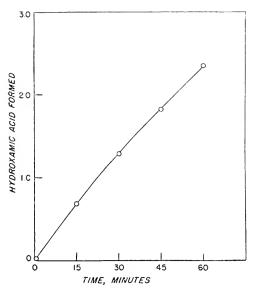


Fig. 3. Time curve of hydroxamic acid formation. Conditions as in Fig. 2, 0.02 M octanoate.

in an earlier table, the ester hydrolysis is much more rapid as the condensation reaction and very soon the tributyrin was split to completion. An appreciable exchange should, however, have been shown by a considerable increase of hydroxamate formation with the ester. The values found (Table VI) are practically identical, due to the presence of nearly equivalent amounts of butyrate during the major part of the incubation period. In the sample with tributyrin, the butyrate obviously originated from hydrolysis.

In similar experiments with equivalent amounts of ethyl and sodium butyrate, similar results were obtained. A slight increase of hydroxamate formation was observed in the earlier part of the incubation period, which evened out, however, with the progress of time. This may be due to a non-enzymatic reaction of the ester with hydroxylamine, recently observed under analogous conditions

by Chantrenne<sup>12</sup> or to a slow enzymatic exchange reaction.

 ${\bf TABLE\ VI}$  comparison of equivalent amounts of tributyrin and butyrate

| $\mu M$ | $\mu \mathrm{M}$ |
|---------|------------------|
| 3.4     | 0.76             |
| 1.7     | 0.33             |
|         |                  |

o.1 ml of hog liver extract in o.5 ml total volume, o.6 M hydroxylamine. The tributyrin was diluted with 9 volumes of 95% ethanol of which o.o1 ml was added. The same amount of ethanol was added to the butyrate sample to equalize conditions.

#### EXPERIMENTS WITH PANCREAS LIPASE PREPARATIONS

In order further to check the ability of lipase to condense carboxyl groups with hydroxylamine we turned to an exploration of the action of pancreas lipase on fatty acid and hydroxylamine. As source of the enzyme, the marketed pancreatine of Parke-Davis was used. The condensation with hydroxylamine was easily observed likewise with pancreas enzyme, although somewhat less actively than with the liver enzyme. Significantly, the chain length optimum was shifted to the longer chains in accordance with the more truly lipatic nature of the pancreas enzyme.

By using an untreated suspension of pancreatine a rather large blank value was obtained. This could, however, be reduced considerably by washing with slightly acid fluid. Generally, not too much activity went into solution in this manner. The residue

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was used as a suspension. In Table VII, the hydroxamic acid formation with dodecanoate is described using various fractions. The results are analogous to those obtained with the liver enzyme.

| TABLE VII  |      |        |      |              |             |
|------------|------|--------|------|--------------|-------------|
| HYDROXAMIC | ACID | FORMED | WITH | PANCREATINE, | Parke-Davis |

| No. | Preparation                                                         | Dodecanoate<br>M | Hydroxamic Acid<br>μΜ |
|-----|---------------------------------------------------------------------|------------------|-----------------------|
| 1   | Orginal Suspension, 5%                                              | 0.01             | 1.39<br>2.32          |
| 2   | Supernatant                                                         | 0.01             | 1.31<br>1.63          |
| 3   | Residue resuspended to volume                                       | 0.01             | 0.16<br>0.74          |
| 4   | Residue resuspended in <sup>1</sup> / <sub>3</sub> original volumec | 0.01             | 0.40<br>1.94          |

0.5 g of pancreatine was suspended in 10 ml water, an aliquot was used in experiment 1. 20 drops of 0.02 molar acetic acid were added and the suspension shaken up. The suspension was centrifuged for half an hour in the cold room. The supernatant was neutralized and used for experiment 2. The residue was resuspended in 0.02 M ammonia buffer with final  $p_{\rm H}$  of 8, and used for experiments 3 and 4.

Each tube contained 0.14 ml of 2 M hydroxylamine buffer of pH 6.6, 0.25 ml enzyme solution and 0.1 ml of 0.1 M dodecanoate or 0.1 ml water. The dodecanoate solution had to be warmed up before addition. Incubation for 66 minutes at 37°; hydroxamic acid determination in alcoholic solution.

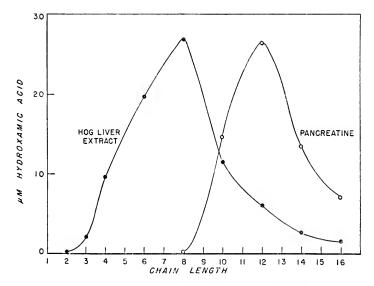


Fig. 4. Chain length optimum for liver and pancreas lipase. The conditions for liver extract were as described in Fig. 3, 60 minutes incubation time. Pancreatine, 5% suspension, 0.75 ml in 0.05 M secondary sodium phosphate, 0.45 ml 2 M hydroxylamine,  $p_H$  7, 0.3 ml of 0.05 M fatty acid salts, 60 minutes incubation.

A survey and comparison of results obtained with the liver and pancreas enzyme appear in Fig. 4. Particularly the difference in the chain length optimum may be noted, the optimum being found at octanoate for liver and at dodecanoate for pancreas lipase. The previously mentioned inhibitory effect of free long-chain fatty acids surely affects somewhat the situation of this optimum. In the experiments with solutions of the salts of higher members of the fatty acid series, the solution was prepared by warming the acid with equivalent amounts of sodium hydroxide. Such solutions jelled on cooling and had to be rewarmed for use in the experiment.

#### DISCUSSION

There are primarily two points that seem to deserve comment; one, the low energy requirement of the hydroxamic acid condensation and two, the apparent non-specificity of this reaction for an esterase. Although no attempts were made here to determine accurately the equilibrium point, it is quite obvious from the relatively low concentration of the reactants which are sufficient to support condensation on the catalyst that the change of free energy with this condensation cannot be more than a few hundred calories. It nevertheless is well known that spontaneous reaction between the free carboxyl group and hydroxylamine will not occur<sup>4</sup> and that therefore hydroxylamine remains to be regarded a trapping reagent for activated carboxyl groups. It is true that such activation need not mean the actual input of considerable energy by a creation of an energy-rich link. However, the acetate<sup>1</sup> or glutamate<sup>13</sup> activation by primary reaction with ATP, so easily measured by use of the hydroxamic acid reaction, bears evidence how valuable a tool hydroxylamine has become for a detection of this type of reaction. Nevertheless as rightly emphasized by Chantrenne<sup>12</sup>, a judicious evaluation of the particular experimental conditions is required and the use of lower concentration of hydroxylamine may be recommended in cases where an activation of carboxyl by primary formation of an energy-rich linkage is suspected.

The "non-specificity" of the here described esterase activity appears of some significance. The link formed here by esterase action may be considered rather a peptidic link. It is thus tempting to look at this reaction as the reverse phenomenon to the esterase activity of chymotrypsin, uncovered recently by Neurath and his group<sup>15</sup>.

#### SUMMARY

A lipase-catalysed condensation of fatty acid and hydroxylamine is described. Reaction in liver extracts follows the inhibition pattern of liver lipase, hexyl resorcinol and fluoride acting as powerful inhibitors. On fractionation of hog liver extract, the esterase and condensation activities remain associated. An analogous reaction is found with pancreatine.

The condensation with hy roxylamine on lipase occurs only with relatively high concentrations of hydroxylamine and the reaction is further enhanced by increase of the fatty acid concentration. To obtain considerable hydroxamic acid formation, the concentration of 0.4 to 0.6 molar of hydroxylamine is required. With liver esterase, the chain length optimum is found with octanoate, while pancreas lipase reacts little with compounds containing below 8 carbons, and shows optimum activity with dodecanoate.

The observations indicate that a relatively small change of free energy occurs with condensation of fatty acids with hydroxylamine to form hydroxamic acid.

For the determination of the hydroxamic acid of long-chain fatty acids, a 50% alcoholic medium is required because of the water insolubility of this compound. The hydroxamic acid determination was modified for 50% ethanol-water.

#### RÉSUMÉ

Les auteurs décrivent une condensation d'acide gras et d'hydroxylamine catalysée par une lipase. La réaction dans les extraits de foie suit le schéma d'inhibition de la lipase de foie, l'hexylresorcine et le fluorure agissant comme inhibiteurs puissants. Lors du fractionnement d'un extrait de foie de porc les activités d'esterase et de condensation restent associées. L'on trouve une reaction semblable pour la pancreatine.

La condensation avec l'hydroxylamine sous l'action de la lipase se produit seulement à des concentrations relativement élevées d'hydroxylamine et elle est accélérée par une augmentation de la concentration en acide gras. Pour obtenir une formation d'acide hydroxamique considerable, l'on doit avoir une concentration 0.4 à 0.6 molaire en hydroxylamine. Avec la lipase de foie l'optimum de longueur de chaine est atteint avec l'octanoate, tandis que la lipase de pancréas reagit peu avec les composés contenant moins de 8 atomes de carbone et montre une activité optima pour le dodé-

Les observations que nous avons pu faire indiquent qu'un changement relativement faible d'énergie libre se produit lors de la condensation des acides gras avec l'hydroxylamine pour former les acides hydroxamiques correspondants.

Pour la détermination des acides hydroxamiques d'acides gras à longue chaîne, il faut employer un milieu contenant 50% d'alcool, parceque ces produits sont insolubles dans l'eau. La determination d'acide hydroxamique a été modifiée pour un milieu éthanol/eau à 50%.

#### ZUSAMMENFASSUNG

Eine durch Lipase katalysierte Kondensation der Fettsäuren mit Hydroxylamin wird beschrieben. Die Reaktion in Leberextrakten folgt dem Hemmungsschema der Leberlipase; Hexylresorcin und Fluorid wirken als starke Hemmstoffe. Bei der Fraktionierung eines Schweineleberextraktes bleiben die Esterase- und Kondensationsaktivitäten vereinigt. Eine analoge Reaktion wurde für Pankreatin gefunden.

Die Kondensation mit Hydroxylamin über Lipase findet nur bei verhältnismässig hohen Hydroxylaminkonzentrationen statt und wird durch Zunahme der Fettsäurekonzentration weiter gesteigert. Zur Erlangung einer erheblichen Hydroxamsäurebildung ist eine o.4 bis o.6 molare Hydroxylaminkonzentration erforderlich. Für Leberlipase ist die optimale Kettenlange mit dem Oktanoat erreicht, wahrend Pankreaslipase nur schwach mit Verbindungen reagiert, die weniger als 8 Kohlenstoffatome enthalten und für das Dodekanoat eine optimale Aktivitat zeigt.

Unsere Beobachtungen weisen darauf hin, dass bei der Kondensation von Fettsäuren mit Hydroxylamin unter Bildung von Hydroxamsäuren verhältnismässig geringe Änderungen der freien Energie stattfinden.

Zur Bestimmung der Hydroxamsäuren von Fettsauren mit langen Ketten muss, wegen der Unlöslichkeit dieser Verbindungen in Wasser, in 50% igem Alkohol gearbeitet werden. Die Hydroxamsäurebestimmung wurde für 50% iges Äthanol/Wasser angepasst.

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# ACYLATION REACTIONS MEDIATED BY PURIFIED ACETYLCHOLINE ESTERASE II\*

by

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The probability that acetylcholine esterase plays a role in the generation of the action potential<sup>1</sup> lends special interest to the study of the nature of this enzyme and of the reactions which it may mediate. In an earlier communication<sup>2</sup> the ability of the electric tissue esterase of *Electrophorus electricus* to mediate acylations of choline and hydroxylamine was noted. In the present report, factors which govern the rate and extent of these reactions are considered.

The specificity and affinity of purified electric tissues esterase for a wide range of substrates and inhibitors have been studied by Nachmansohn et al.<sup>3</sup>, <sup>4</sup> and more recently by Augustinsson<sup>5</sup>, <sup>6</sup>. An important function of the enzyme — the hydrolysis of esters as a function of p<sub>H</sub>—has not been described previously. The manometric method of esterase assay is conveniently applicable within a narrow range of p<sub>H</sub>. Characterization of the p<sub>H</sub> function of the enzyme by the potentiometric technique for the determination of the acid reaction product would be feasible but laborious. A colorimetric method<sup>7</sup> for the assay of ester in the presence of excess of products of ester hydrolysis affords a convenient procedure for assay of esterase activity at any desired p<sub>H</sub>. The method is applicable equally to measurement of both hydrolysis and synthesis of the ester and with its aid information concerning the p<sub>H</sub> function of an esterase is easily obtainable.

#### METHODS

Acetycholine and propionylcholine were determined according to the procedure previously described? Aliquots of 0.5 or 1.0 ml of the test solution containing 0.3 to 4.0  $\mu\rm M$  of ester were used for the determinations.

Acethyldroxamic and propionlydroxamic acid were measured in aliquots of 0.5 or 0.1 ml containing 0.3 to 4.0  $\mu$ M. The samples were brought to p<sub>H</sub> 1.0–1.4 with hydrochloric acid and then estimated colorimetrically with 1% ferric chloride essentially as in the method for the determination of acetylcholine?

The Klett photoelectric colorimeter was used with green filter 54.

#### Enzyme

Acetylcholine esterase of the electric tissue of *Electrophorus electricus* was used. The enzyme was purified according to the method described by Rothenberg and Nachnansohn<sup>8</sup>. The enzyme was dissolved in a medium of sodium phosphate 0.05 M, magnesium chloride 0.02 M, and sodium chloride 0.1 M at ph 7.0 and stored in the cold at 4°C. Stock enzyme solutions were diluted into 2.8% gelatin freshly before use. In the hydrolysis experiments the final dilution of the enzyme solution was in the order of magnitude of one part in ten thousand; in the experiments on acylation a much higher enzyme concentration — an order of magnitude of one part in ten — was used.

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<sup>\*</sup> This work has been carried out under grants from the U. S. Public Health Service and the Office  $\epsilon$ f Naval Research.

#### A. HYDROLYSIS OF ACETYLCHOLINE AS A FUNCTION OF PH

An enzyme concentration assay curve is reproduced in Fig. 1. The hydrolysis-time curves in phosphate solution at  $p_{\rm H}$  7.4 depart from a straight line to a measurable extent only after about 30% of the substrate at an initial concentration of 4  $\mu M$  per ml has been split. The plot of the initial reaction velocity against enzyme concentration in the range studied yields a straight line.

Fig. 1. Acetylcholine hydrolysis as a function of enzyme concentration. Mixtures contain 1.0 M potassium dihydrogen phosphate adjusted with sodium hydroxide to pH 7.4, gelatin 0.07%, acetylcholine μM/ml. Temperature 23°C. The pH remained constant within 0.2 pH nnits during the course of the hydrolysis. The non-enzymatic hydrolysis in these conditions was barely detectable. Curves 1–5 show findings with enzyme dilutions 1:4000, 1:8000, 1:12000, 1:20000 and 1:30000 respectively. In the inset, relative enzyme concentration is plotted on the abscisca and the corresponding relative initial reaction velocity on the ordinate.

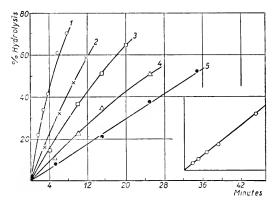


TABLE I

ACETYLCHOLINE HYDROLYSIS IN PHOSPHATE SOLUTION AS A FUNCTION OF PH IN THE ACID RANGE

The solutions contained a constant amount of enzyme, 0.07% gelatin, 0.1 M potassium phosphate, sodium hydroxide in varying amounts and acetylcholine chloride in a concentration of 4  $\mu$ M/ml. The pH remained constant during the course of the hydrolysis within 0.2 pH units. Temperature 21° C. Non-enzymatic hydrolysis proved negligible in the conditions used. Control mixtures to which no acetylcholine was added failed to produce colour when examined with the reagent. The solutions remained clear and removal of the protein present in the reaction mixture was unnecessary.

|            | Per cent hydrolysis at times (min) |     |     |     |  |  |
|------------|------------------------------------|-----|-----|-----|--|--|
| РН         | 10'                                | 20' | 30' | 40′ |  |  |
| 7.8        | 17                                 | 32  | 45  | 57  |  |  |
| 7·4<br>6.8 | 16                                 | 31  | 45  | 55  |  |  |
|            | 15                                 | 28  | 39  | 49  |  |  |
| 6.3<br>5.8 | 13                                 | 25  | 35  | 44  |  |  |
| 5.8        | 9                                  | 17  | 23  | 30  |  |  |
| 5.5        | _                                  | 13  | _   |     |  |  |

Variation of esterase activity accompanied shift of  $p_H$  on the acid side of the scale in a range which is still of physiological interest. The course of the reaction in phosphate buffer is illustrated by the experiment recorded in Table I. It is evident that increase of  $p_H$  from 5.5 to 7.4 results in a progressive and marked rise of reaction rate in phosphate buffer. Between  $p_H$  7.4 and 7.8 in phosphate and between  $p_H$  7.6 and 9.4 in borate the enzyme-mediated hydrolysis exhibited a constant initial reaction rate. At  $p_H$  higher than 9.4 inactivation of enzyme occurred at 21° C., the inactivation was retarded considerably at 17° C. Non-enzymatic hydrolysis of the substrate was found to become relatively appreciable at  $p_H$  9.2 and rose rapidly with further increase of the  $p_H$  (Table II). A summary of findings is presented in Fig. 2. The  $p_H$  range in which the acetylcholine References  $p_H$  321.

#### TABLE II

ACETYLCHOLINE HYDROLYSIS IN BORATE SOLUTION AS A FUNCTION OF PH IN THE ALKALINE RANGE

a) Reaction mixtures contained a constant amount of enzyme, acetylcholine chloride 4  $\mu$ M/ml., 0.07% gelatin and 2 ml of Sörensen borate buffer in 4 ml of final mixture. Temperature 21° C. p<sub>H</sub> remained unchanged within 0.2 p<sub>H</sub> units throughout the course of reaction. Non-enzymatic hydrolysis was negligible.

| -1 |             |                      |                               |                                       |
|----|-------------|----------------------|-------------------------------|---------------------------------------|
| 3  | 10′         | 20′                  | 30'                           | 35′                                   |
| 8  | 17          | 30                   | 43                            | 51                                    |
| 8  | 18          | 31                   | 44                            | 49<br>50                              |
|    | 8<br>8<br>9 | 8 17<br>8 18<br>9 18 | 8 17 30<br>8 18 31<br>9 18 33 | 8 17 30 43<br>8 18 31 44<br>9 18 33 — |

b) As in a) but with borate-potassium chloride-sodium carbonate solutions of Atkins and Pantin<sup>11</sup> as the buffer. Enzyme was added to the reaction mixtures as the last component. By use of a high enzyme concentration and a rather low temperature for the incubation the relative role of the non-enzymatic hydrolysis could be kept to a minimum. The same device served also to prevent undue interference at highly alkaline  $p_H$  by progressive inactivation of the enzyme. The temperature was  $17^{\circ}$  C.  $p_H$  remained unchanged within 0.2  $p_H$  units throughout the observed course of the reaction.

|                             |               | Pe               | rcentage hy          | drolysis at       | times (mir    | 1)           |                    |
|-----------------------------|---------------|------------------|----------------------|-------------------|---------------|--------------|--------------------|
| РН                          | 3'            | 4'               | 6′                   | 8'                | 9′            | 10'          | 12'                |
| Total hydrolysis            |               |                  |                      |                   |               |              |                    |
| 8.5<br>9.4<br>10.0<br>10.4  | 17<br>15<br>— | 2I<br>22         | 30<br>28<br>31<br>31 | 38<br>39          | 42<br>41<br>— | <br>45<br>44 | 53<br>50<br>—      |
| Non-enzymatic<br>hydrolysis |               |                  |                      |                   |               |              |                    |
| 8.5<br>9.4<br>10.0<br>10.4  |               | 2<br>0<br>5<br>6 | <br><br>             | 0<br>1<br>9<br>11 |               | —<br>—<br>—  | 0<br>2<br>13<br>16 |
| Enzymatic<br>hydrolysis     |               |                  |                      |                   |               |              |                    |
| 8.5<br>9.4<br>10.0<br>10.4  | 15<br>15<br>— | 16<br>16         | 30<br>28<br>24<br>22 |                   | 42<br>40<br>— | 34<br>31     | 53<br>48<br>—      |

hydrolysis was essentially independent of  $p_H$  is relatively wide. The  $p_H$  function of the acetylcholine esterase from electric tissue differs in this respect from some other esterases which have been studied by  $GLICK^9$ .

The effect of addition of choline and acetate on acetylcholine hydrolysis has been studied in detail by Augustinsson<sup>10</sup>. It seemed of interest to ascertain whether  $p_H$  influences the role played by the hydrolysis products. In an experiment reported in Table III the effect of choline chloride (12.5  $\mu$ M/ml) on the hydrolysis of acetylcholine (4  $\mu$ M/ml) at three selected  $p_H$  values is shown. Choline proved to be about equally inhibiting at  $p_H$  7.7 and 6.8; the choline was only about one-half as active an inhibitor at  $p_H$  5.9. Acetate even in high concentrations (0.1 M) failed to inhibit acetylcholine hydrolysis by electric tissue esterase in phosphate solution either at  $p_H$  5.5 or 7.7. Since

moderation of the action of esterase inhibitors by way of regulation of  $p_H$  might be a matter of some practical as well as theoretical interest, further study of the  $p_{H^-}$  dependence of esterase-inhibitor inter-

actions appears desirable.

Fig. 2. Acetylcholine hydrolysis as a function of p<sub>H</sub>. Curve 1: Hydrolysis of acetylcholine in the presence of enzyme. Relative initial reaction rates corrected for enzymatic hydrolysis are plotted on the ordinate. The curve is a composite of data given in Tables I and II. Values for p<sub>H</sub> 7.8 in phosphate, and p<sub>H</sub> 8.1 and 8.5 in borate are taken equal to 10. Curve 2: Hydrolysis of acetylcholine in absence of enzyme. Acetylcholine concentration 4 \mu M/ml. p<sub>H</sub> was regulated with borate buffer. Initial reaction rates are plotted on the ordinate. The value for p<sub>H</sub> 10.6 is taken equal to 10. The temperature was 21° C.

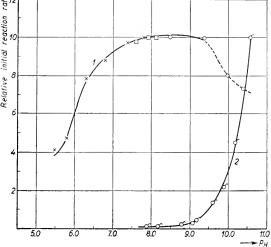


TABLE III

INFLUENCE OF CHOLINE ON ACETYLCHOLINE HYDROLYSIS AT DIFFERENT PH VALUES

Reaction mixtures contained a constant amount of enzyme, acetylcholine chloride 4  $\mu$ M/ml, choline chloride (or sodium chloride) 12.5  $\mu$ M/ml, potassium phosphate 0.1 M, sodium chloride 0.05 M, magnesium chloride 0.02 M, gelatin 0.07% and different amounts of sodium hydroxide. Temperature 37° C.

| _      | Choline |     | ]   | Percent hy | irolysis at | times (min | )   |     |
|--------|---------|-----|-----|------------|-------------|------------|-----|-----|
| PH Cho | Chonne  | 10' | 20′ | 30′        | 40′         | 50'        | 60' | 70' |
| 7.7    |         | 20  | 40  | 58         | 71          |            | _   | _   |
| 7.7    | +       | _   | 15  | 22         | 32          | 39         | _   | _   |
| 6.8    |         | 20  | 38  | 53         | 65          |            | _   | _   |
| 6.8    | +       |     | 15  | 21         | _           | 39         | _   | _   |
| 5.9    | _       | _   | _   | 22         |             | 38         | _   | 49  |
| 5.9    | +       |     | _   | 16         | _           | 26         | _   | 38  |
|        |         |     |     |            |             |            |     |     |

### B. SYNTHESIS OF ACETYL- AND PROPIONYLCHOLINE BY THE ACTION OF PURIFIED ACETYLCHOLINE ESTERASE

The equilibrium constant of esterification reactions favours strongly the reaction direction of hydrolysis<sup>12</sup>. Earlier investigators<sup>13</sup> observed that the pharmacological activity of choline is enhanced by incubation with acetate in the presence of crude tissue preparations of esterase. Demonstration of this synthesis and measurement of the equilibrium was greatly facilitated in the present work by the availability of the hydroxylamine method which could be applied to the determination of the ester in the presence of a large excess of the products of the hydrolysis.

Figs 3 and 4 analyse the effect of  $p_H$  on the equilibrium position of the hydrolysis of acetylcholine and propionylcholine respectively by the purified esterase. The approach to equilibrium at three selected  $p_H$  was realized in each case from both reaction directions.

References p. 321.

It is apparent that acid shift of  $p_H$  within the range studied displaces the equilibrium in the direction of synthesis. In the experiments of Figs 3 and 4 the speed of the approach to the equilibrium was found to be dependent upon the esterase concentration. To insure a close approach to the equilibrium in a conveniently short time, a much greater enzyme concentration than is conveniently used in a hydrolysis assay was taken.

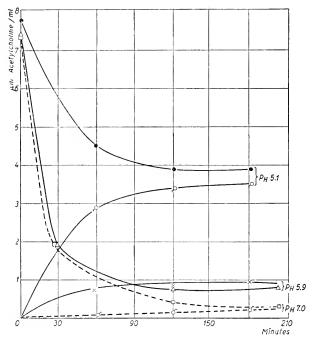


Fig. 3. Synthesis of acetylcholine as a function of p<sub>H</sub>. Solutions were made with 1.15 g each of choline chloride and sodium acetate trihydrate at p<sub>H</sub> 5.1 in a total volume of 6.0 ml, and with 1.21 g each of these substrates in the same total volume at p<sub>H</sub> 5.9 and 7.0. p<sub>H</sub> was set with hydrochloric acid and measured with a glass electrode in samples diluted for the purpose with three volumes of water. In one control mixture at each p<sub>H</sub>, 8 µM of acetylcholine per ml was added at the outset. Enzyme was added in an amount per ml sufficient to effect hydrolysis of 2 g of acetylcholine chloride per hr in optimum conditions. Temperature 23° C. Ester was determined on aliquots of 0.5 ml. A standard curve was constructed with known acetylcholine amounts in the same medium. Care is taken in the ester determination to bring the p<sub>H</sub> of the sample to 1.0–1.2 at the step prior to ferric chloride addition in order to avoid interfering colour by reaction between fatty acid and ferric chloride. In several cases, water was added to a reaction mixture in which the synthesis had come to a rest. A rapid shift of the equilibrium in the reaction direction of hydrolysis could then be observed. In the absence of either acetate, choline, or esterase, no ester formation was observed.

|     | Concentration at equilibrium (molarity) |         |                             |                |          | K         | ΔF                                             |
|-----|-----------------------------------------|---------|-----------------------------|----------------|----------|-----------|------------------------------------------------|
| Рн  | water                                   | choline | acetic acid<br>plus acetate | acetic<br>acid | acetyl-  | _ (a · e) | $= -4.58 \text{ T} \log \frac{55.5}{\text{K}}$ |
|     | (a)                                     | (b)     | (c)                         | (d)            | (e)      | (b·d)     | = 4.30 T log K                                 |
| 5.1 | 41                                      | 1.35    | 1.4                         | 0.45           | 3.7.10-3 | 0.25      | -3 160                                         |
| 5.9 | 39                                      | 1.45    | 1.5                         | 0.1            | 1.0.10-3 | 0.27      | -3140                                          |

The effect of  $p_H$  on the equilibrium might be interpreted as follows. On general grounds, it seems reasonable to suppose that the immediate product of ester hydrolysis is the undissociated acid molecule rather than its ion:

$$RCOOR' + H_2O \Rightarrow RCOOH + R'OH$$
 (1)

$$RCOOH \Rightarrow RCOO^- + H^+$$
 (2)

where RCOOR' represents the ester and RCOOH and R'OH the acid and alcohol products of hydrolysis. Equilibrium in the synthesis will then be defined by the relationship:

$$K = \frac{[H_2O] [RCOOR']}{[R'OH] [RCOOH]}$$

where K is the Nernst equilibrium constant calculated from concentrations in molarity. As p<sub>H</sub> is decreased, the concentration of the undissociated acid rises and an accompanying

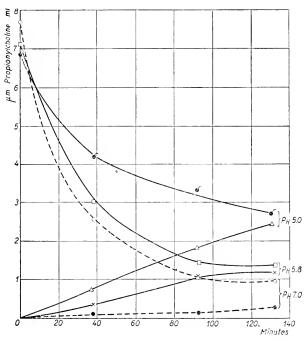


Fig. 4. Synthesis of propionylcholine as a function of  $p_H$ . Solutions were made with 1.21 g of choline chloride and 0.85 g of sodium propionate at  $p_H$  7 and 5.8 in a total volume of 6.0 ml, and with 1.01 g of choline chloride and 0.71 g of sodium propionate at  $p_H$  5.0 in the same total volume. Temperature 18° C. Procedure otherwise as described under Fig. 3.

|     | Concentration at equilibrium (molarity) |         |                                   |      |             |                                                                        | ΔF                                              |
|-----|-----------------------------------------|---------|-----------------------------------|------|-------------|------------------------------------------------------------------------|-------------------------------------------------|
| РН  | water                                   | choline | propionic acid<br>plus propionate |      |             | $=\frac{(\mathbf{a} \cdot \mathbf{e})}{(\mathbf{b} \cdot \mathbf{d})}$ | $= -4.58 \text{ T } \log \frac{55.5}{\text{K}}$ |
|     | (a)                                     | (b)     | (c)                               | (d)  | (e)         | (D.d)                                                                  | K                                               |
| 5.0 | 41                                      | 1.2     | 1.25                              | 0.52 | 2.6 · 10-3  | 0.17                                                                   | -3350                                           |
| 5.8 | 39                                      | 1.45    | 1.5                               | 0.15 | 1.26 · 10-3 | 0.23                                                                   | -3170                                           |

increase of ester concentration at equilibrium may be expected. The values found for the K of the choline esterifications approximated 0.2 within the limits of the experimental error\*. The reasonably good constancy of the values for K despite the large variation of the absolute concentration of ester at equilibrium in the investigated  $p_H$  range supports the suggestion that undissociated acid rather than the anion enters into the equilibrium of the esterification.

A value for the  $\Delta$  F of choline ester hydrolysis may be calculated from K with the aid of the relationship

$$-\Delta F = RT \ln \frac{55.5}{K}$$

whose derivation has been discussed recently by Meyerhof and Green<sup>14</sup>. —  $\Delta$  F calculated in this manner was found to approximate 3200 cals. Although molarities rather than activities are used above to calculate K, it is believed likely that error from this cause in the value for  $\Delta$  F does not exceed 10% \*\*. It is noteworthy that the value for  $\Delta$  F of hydrolysis of two choline esters is of an order similar to the observed in the case of several anionic esters<sup>14</sup>.

The amount of the acetylcholine at equilibrium is minute in comparison to the concentration of the other participants of the system. However, it seems desirable in view of the great biological potency of acetylcholine to consider the possibility that esterase functions as an agent of acetylcholine synthesis in vivo, supplementing in this respect the role of choline acetylase. It has been demonstrated that acetylcholine esterase in the nerve axon is localized in the neuronal surface membranes <sup>15</sup>. The concentration of esterase substrates and the  $p_H$  prevailing in the membrane are unknown, but there is reason to believe that H<sup>+</sup> and choline<sup>+</sup> may be significantly higher at the membrane interface than in the surrounding milieu<sup>16</sup>. Specific binding of ester and sudden variation in  $p_H$  at the membrane with resulting shift of equilibrium are conceivable. For a local choline concentration of 0.01 M and a similar concentration of undissociated acetic acid, the value 0.2 for K leads to an equilibrium acetylcholine concentration of 0.06 micrograms per ml. An ester concentration of this order would be sufficient to produce major biological effects.

#### C. FORMATION OF HYDROXAMIC ACIDS

The ability of proteolytic enzymes to catalyse ester hydrolyses has been demonstrated by Neurath and his coworkers<sup>17</sup>. The ability of O-acyl hydrolases-lipase<sup>18</sup> and esterase<sup>2</sup> to form hydroxamic acids by the condensation of fatty acid with hydroxylamine is an interesting counterpart to this situation in which a group of hydrolases catalyses both O- and N-acylation.

The effect of reactant concentrations on the rate of the formation of hydroxamic acid in the presence of the electric tissue esterase is shown by experiments summarized in Fig. 5. Within a wide range of reactant concentration the relation between reaction rate and reactant concentration remains almost linear. Reactant concentrations up to 0.75 M or higher failed to saturate the enzyme. Its affinity for acetate, propionate, and

\*\* I am much indebted to Professor O. MEYERHOF for the discussion of this question.

<sup>\*</sup> Inaccuracy in the measurement of  $p_H$  would exert a relatively large effect on the value of K. The computation of K for  $p_H$  above 6 suffers from an additional inaccuracy because the concentration of ester approached the limit of the ester determination as the  $p_H$  increased above 6.

hydroxylamine may be concluded, therefore, to be of a much lower order than the affinity of the enzyme for acetylcholine. This conclusion has been further supported by the demonstration that neither acetate nor hydroxylamine significantly affect the rate of acetylcholine hydrolysis by the esterase. The substrate concentration-activity relationship observed in hydroxylamine acylation resembles that of neutral ester hydrolysis by the enzyme<sup>3, 5</sup>.

The rate of reaction of acetate with hydroxylamine in the presence of esterase is very small as compared to the rate of hydrolysis of acetylcholine by a similar concentration of the enzyme, the relative magnitude of the rates being in the proportion of one to one or two thousand. The rate of hydroxamic acid formation, like the hydrolysis of acetylcholine, varied in a direct manner with the esterase concentration (see Fig. 6).

The specificity of electric tissue esterase in regard to the fatty acids which it can cause to condense with hydroxylamine is rather sharply defined (see Table IV). As in choline ester hydrolysis<sup>3</sup>, a maximum is observed with acetic acid. A lower rate is found with propionic acid. The enzyme-catalyzed reaction observed with butyric acid was almost negligible. The findings with formic acid reveal a relatively large spontaneous reaction between formate and

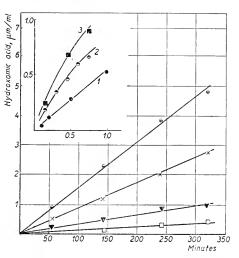


Fig. 5. Formation of hydroxamic acid as a function of reactant concentration. The reaction mixtures are 0.5 M as to sodium acetate and 1.0 M as to sodium chloride.  $p_H$  6.8.  $37^{\circ}C$ .  $\square$ ,  $\nabla$ ,  $\times$ ,  $\bigcirc$ , — correspond to mixtures with 0.1, 0.2, 0.5, and 1.0 M hydroxylamine respectively. Curves 1 to 3 of the inset are not mutually comparable since they were obtained with different batches of the enzyme. Relative reaction rates are plotted on the ordinates and reactant concentrations in molarity on the abscissae. Curve I summarizes the detail of the main part of the figure showing the effect of variation of hydroxylamine concentration. Curves 2 and 3 show the effect of variation of acetate and propionate concentration respectively in the presence of 1.0 M hydroxylamine.

TABLE IV

SUBSTRATE SPECIFICITY OF ELECTRIC TISSUE ESTERASE IN FORMATION OF HYDROXAMIC ACID

The reaction mixtures are 1.0 M as to hydroxylamine and 0.75 M as to the sodium salt of the fatty acid in 0.9 M solution of sodium chloride at  $p_{\rm H}$  6.2–6.4. Temperature  $37^{\circ}$  C. Propionhydroxamic, butyrhydroxamic, and acethydroxamic acid yield equivalent amounts of colour per mole with ferric chloride. The amount of the formhydroxamic acid is calculated on the same basis.

| Fatty acid | Hydroxamic acid, μM/ml, at times in minutes                                      |                                                                                                         |                                                                                                                                                                                                                                                                                                                                                              |  |
|------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
|            | 50                                                                               | 100                                                                                                     | 200                                                                                                                                                                                                                                                                                                                                                          |  |
| formate    | 1.3                                                                              | 2.4                                                                                                     |                                                                                                                                                                                                                                                                                                                                                              |  |
| formate    | 0.7                                                                              | 1.3                                                                                                     |                                                                                                                                                                                                                                                                                                                                                              |  |
| acetate    | 3.0                                                                              | 6.0                                                                                                     | 9.9                                                                                                                                                                                                                                                                                                                                                          |  |
| acetate    | 0.0                                                                              | 0.0                                                                                                     | 0.1                                                                                                                                                                                                                                                                                                                                                          |  |
| propionate | 1.1                                                                              | 2.3                                                                                                     |                                                                                                                                                                                                                                                                                                                                                              |  |
| propionate | 0.0                                                                              | 0.0                                                                                                     |                                                                                                                                                                                                                                                                                                                                                              |  |
| butyrate   |                                                                                  | 0.3                                                                                                     | 0.6                                                                                                                                                                                                                                                                                                                                                          |  |
| butyrate   |                                                                                  | 0.2                                                                                                     | 0.5                                                                                                                                                                                                                                                                                                                                                          |  |
|            | formate<br>formate<br>acetate<br>acetate<br>propionate<br>propionate<br>butyrate | Fatty acid at t  50  formate 1.3 formate 0.7 acetate 3.0 acetate 0.0 propionate 1.1 propionate butyrate | Fatty acid         at times in min           50         100           formate         1.3         2.4           formate         0.7         1.3           acetate         3.0         6.0           acetate         0.0         0.0           propionate         1.1         2.3           propionate         0.0         0.0           butyrate         0.3 |  |

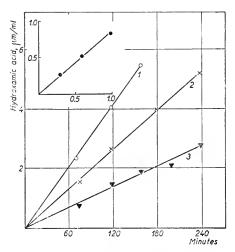


Fig. 6. Formation of hydroxamic acid as a function of esterase concentration. Reaction mixtures were 1.0 M as to hydroxylamine and sodium acetate in 1.0 molar sodium chloride. pH 6.8. 37° C. Curves 1 to 3 correspond to relative enzyme concentrations 10, 6, and 3. In the inset the relative reaction rate is plotted on the ordinate and the relative enzyme concentration on the abscissa.

hydroxylamine, and some enzymatic catalysis of this reaction. Substitution of an  $\alpha$ -amino group into acetate or propionate caused complete loss of their ability to condense with hydroxylamine in the presence of the enzyme.

The ability of the esterase to effect hydrolysis of acethydroxamic acid was examined at a substrate concentration of 3  $\mu$ M/ml in phosphate buffer at p<sub>H</sub> 7.4. Even with a great concentration of enzyme no hydrolysis of acethydroxamic acid was found, although acetylcholine added to the same reaction mixture was hydrolysed rap'dly. An acyl transfer reaction between acethydroxamic acid and choline with resultant intermediary formation of hydrolysable acetylcholine could be excluded, since addition of choline to the same reaction mixture failed to evoke a disappearance of acethydroxamic acid.

The  $p_H$  dependence of hydroxylamine acylation by electric tissue esterase is illustrated by the experiment given in Table V. The reaction between acetate and hydroxylamine showed a peak in a range near  $p_H$  6.3. The  $p_H$  function of hydroxylamine acylation

by the esterase is thus very different from the  $p_H$  function of acetylcholine hydrolysis by the enzyme. The finding that the  $p_H$  dependence of hydroxylamine acylation and choline ester hydrolysis are quite different is consistent with an assumption, discussed later, concerning the mechanism of these two reactions.

#### TABLE V

FORMATION OF HYDRONAMIC ACID IN PRESENCE AND ABSENCE OF CHOLINE AT DIFFERENT  $p_H$  Reaction mixtures are 0.5 M as to acetate and 1.0 M as to hydroxylamine in 0.9 M solution of sodium chloride at  $p_H$  specified with or without addition of 0.5 M choline chloride. In absence of choline addition, an equivalent amount of sodium chloride was added. The  $p_H$  was determined in aliquots with a glass electrode after four-fold dilution with water. Temperature 37° C. The formation of hydroxamic acid in absence of enzyme was negligible at  $p_H$  6.3 and 5.3 and none was detected at  $p_H$  7 and higher. The reaction time was 4 hours.

| РН   | Choline | Hydroxamic<br>acid, μM/ml |
|------|---------|---------------------------|
| 7.9  | +       | 0.2                       |
| 7.5  |         | 1.5                       |
| 7. I | +       | 0,2                       |
| 7.1  |         | 1.6                       |
| 6.3  | +       | 0.7                       |
| 6.3  | _       | 2.4                       |
| 5.3  | +       | 0.4                       |
| 5.3  |         | 0.2                       |

In the presence of choline, the rate of the acetylation of hydroxylamine by esterase References p. 321.

acting at p<sub>H</sub> 7.1 was reduced markedly (Fig. 7). The by the experiment shown in Table v. As ... case of acetylcholine hydrolysis, the lowering of p<sub>H</sub> be inhibitory effect of choline. At p<sub>H</sub> 5.3, § 7 low but still measurable, an activating effect by choline on hydroxamic acid formation was observed. The inhibitory effect of choline can be ascribed to its ability to combine with the enzyme at an active site<sup>10</sup>. An explanation of activation by choline may be found in the fact that at acid p<sub>H</sub> the concentration of acetylcholine in the system is increased. It has been shown<sup>7</sup> that acetylcholine acetylates hydroxylamine rapidly at alkaline  $p_H$  and slowly at acid pH, the rate being dependent on the concentration of the acetylcholine at constant hydroxylamine concentration. At p<sub>H</sub> 7 the concentration of acetylcholine in the acetate-hydroxylaminecholine-system is neglig ble. The ability of choline to serve as an acetyl carrier at this p<sub>H</sub> must therefore become very small.

The inhibitory effect of choline on hydroxylamine acylation and the finding<sup>2</sup> that incubation of the enzyme with specific inhibitors—prostigmine and tetraethylpyrophosphate—abolishes the abi-

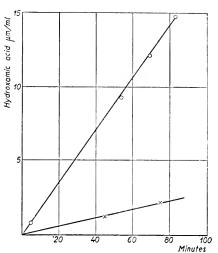


Fig. 7. Effect of choline on formation of hydroxamic acid. The reaction mixtures are 1.0 M as to hydroxylamine and 0.75 M as to sodium acetate in 0.9 M solution of sodium chloride at pH 7.1. Temperature 37° C. O, mixture without choline; X, mixture with 0.9M choline chloride. The reaction in absence of enzyme is negligible in both cases. The inhibitory effect of choline was unaffected by the choline concentration in the range of 0.1 to 0.9 M.

lity to catalyse hydroxamic acid formation support the view that the same enzyme, and possibly the same prosthetic group, effects both acetylcholine hydrolysis and hydroxamic acid formation. But the reaction of hydrolysis of ac tylcholine is reversible, while that of hydroxylamine acylation appears to be irreversible. Choline shows a fairly marked affinity for the enzyme, whereas hydroxylamine shows little or no affinity. The possibility has therefore to be considered that the role of esterase in hydroxylamine acylation is confined to the activation of the carboxylic acid reactant, and that a terminal reaction between activated carboxylic acid and hydroxylamine is spontaneous and irreversible. In the case of choline acylation it is assumed that the esterase may activate the two reactants.

The writer is deeply indebted to Professor D. Nachmansohn for encouragement and for many suggestions. Thanks are expressed to Mrs Emily Feld Hedal and Miss Louise d'Alessio for their assistance in the performance of the experiments.

#### SUMMARY

1. Some general properties of ester hydrolysis and synthesis by the purified acetylcholine esterase of the electric tissue of *Electrophorus electricus* have been investigated with the aid of a simple colorimetric technique for the determination of an ester in the presence of its hydrolysis products.

2. The hydrolysis of acetyl- and propionylcholine by the esterase have been shown to be reversible. The equilibrium of the reaction was found to be characterized by the ratio:

$$\frac{\text{[acetylcholine] [water]}}{\text{[choline] [RCOOH]}} = K$$

where RCOOH represents the undissociated form of the carboxylic acid.

- 3. The possibility that esterase plays a part in synthesis of acetylcholine at the neuronal membrane surface has been discussed.
- 4. The condensation of fatty acids with hydroxylamine by the action of the esterase has been investigated in respect to its dependence on reactant concentration, enzyme concentration, carboxylic acid structure, and  $p_{\rm H}$ .
- 5. Acethydroxamic acid was not hydrolysed by the esterase either in the presence or absence of choline. The reaction of hydroxamic acid formation, unlike ester hydrolysis by the enzyme, thus appear to be irreversible.

6. Condensation of acetate with hydroxylamine in the presence of esterase acting at  $p_{H}$  6.3

and above was markedly inhibited by choline.

7. A reaction mechanism which could explain some of the differences observed between the catalysis of choline ester hydrolysis and that of hydroxamic acid formation by the same esterase has been discussed.

#### RÉSUMÉ

- 1. Quelques propriétés générales de l'hydrolyse et de la synthèse des esters par l'acétylcholine estérase purifié du tissu électrique de *Electrophorus electricus* ont été étudiées à l'aide d'une technique colorimétrique pour la détermination d'un ester en présence de ses produits d'hydrolyse.
- 2. On a montré que l'hydrolyse de l'acétylcholine et de la propionylcholine par l'estérase est réversible. L'équilibre de la réaction est caractérisé par le quotient:

$$\frac{\text{[ac\'{e}tylcholine] [eau]}}{\text{[choline] [RCOOH]}} = K$$

où K représente la forme non dissociée de l'acide carboxylique.

- 3. La possibilité que l'estérase joue un rôle dans la synthèse de l'acétylcholine à la surface de la membrane neuronale a été discutée.
- 4. La condensation des acides gras avec l'hydroxylamine sous l'action de l'estérase a été étudiée en ce qui concerne sa dépendance de la concentration de la substance réagissante et de l'enzyme, de la structure de l'acide carboxylique et du  $p_H$ .
- 5. L'acide acétylhydroxamique n'a pas été hydrolysé par l'estérase ni en présence ni en absence de choline. Ainsi la formation de l'acide hydroxamique, contrairement à l'hydrolyse d'un ester par l'enzyme, semble être irréversible.
- 6. La condensation d'acétate avec l'hydroxylamine en présence d'estérase à un p<sub>H</sub> de 6.3, est

considérablement inhibée par la choline.

7. Un mécanisme de réaction a été discuté qui pourrait expliquer certaines différences observées entre l'hydrolyse d'un ester cholinique et la formation d'acide hydroxamique catalysées par la même estérase.

#### ZUSAMMENFASSUNG

- I. Einige allgemeine Eigenschaften der Esterhydrolyse und -synthese durch gereinigte Acetylcholinesterase aus dem elektrischen Gewebe von *Electrophorus electricus* wurden untersucht und zwar mit Hilfe einer einfachen kolorimetrischen Arbeitstechnik zur Bestimmung eines Esters in Gegenwart seiner Hydrolyseprodukte.
- 2. Es wurde gezeigt dass die Hydrolyse von Acetyl- und Propionylcholin durch die Esterase reversibel ist und dass das Reaktionsgleichgewicht durch den Quotienten

$$\frac{[Acetylcholin] [Wasser]}{[Cholin] [RCOOH]} = K$$

charakterisiert ist, wo RCOOH die nicht dissoziierte Form der Carbonsäure darstellt.

3. Die Möglichkeit wurde erörtert, dass Esterase bei der Acetylcholin-Synthese an der Oberfläche der Neuronmembrane eine Rolle spielen könnte.

4. Die Kondensation von Fettsäuren mit Hydroxylamin unter der Einwirkung der Esterase wurde in Bezug auf die Abhängigkeit dieser Reaktion von der Konzentration der reagierenden Substanz und des Enzyms; sowie von der Struktur der Carbonsäure und dem p<sub>H</sub> untersucht.

- 5. Acetylhydroxamsäure wurde durch die Esterase weder in Gegenwart noch in Abwesenheit von Cholin hydrolysiert. Es scheint also, dass die durch das Enzym katalysierte Hydroxamsäurebildung zum Unterschied von der Esterhydrolyse irreversibel sei.
- 6. Die Kondensation von Acetat mit Hydroxylamin in Gegenwart von Esterase bei p<sub>H</sub> 6.3 wurde durch Cholin stark gehemmt.
- 7. Ein Reaktionsmechanismus, welcher einige Unterschiede zwischen der katalytischen Cholinesterhydrolyse und der Hydroxamsäurebildung unter Einwirkung derselben Esterase erklären könnte, wurde erörtert.

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## OBSERVATIONS ON A FACTOR DETERMINING THE METABOLIC RATE OF THE LIVER

by

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In a paper published some years ago¹ brief mention was made of experiments on isolated, artificially perfused livers in which the rate of oxygen uptake in the liver was consistently found to decrease during the first 30–45 minutes after the liver had been isolated. This phenomenon has intrigued me ever since, and although the cause of this drop in metabolic rate in a liver isolated from the "periphery" is not ascertained a short appraisal of the experience gained so far may be presented.

Most of the experiments have been carried out on cat livers. The metabolism of the isolated cat liver is peculiar in that carbohydrates are not metabolized2. The respiratory quotient of the isolated cat liver is always very low — generally below 0.7. The blood sugar concentration never decreases. Irrespectively of the blood sugar level a steady increase in blood sugar concentration is observed. This increase must be due to a gluconeogenesis as it is observed also in livers in which the glycogen store has been exhausted by starvation. It appears most likely that the lack of carbohydrate metabolism in the isolated cat liver is not an artefact but a characteristic feature in the liver metabolism of this species. Nevertheless one might claim that a liver which does not metabolize carbohydrate must be in an abnormal state and that the drop in metabolic rate might have some connection with this abnormal state. Contrary to the cat liver the isolated rabbit liver, however, stores glucose as glykogen and oxidizes carbohydrate and although my experience with the rate of oxygen consumption in the isolated rabbit liver is far more limited than my experience with cat livers it can safely be stated that in the isolated rabbit liver also a drop in metabolic rate is encountered immediately after isolation.

It might well be questioned whether any importance can be attached to a drop in metabolic rate in an organ kept alive by artificial perfusion. Such a view appears justified, however, since such a decline in oxygen uptake is observed in experiments on livers only and not in experiments on other organs. In the—unfortunately unsuccessful—endeavour to make preparations of isolated cat intestines function normally with respect to absorption a considerable number of experiments have been carried out in which the oxygen uptake of the isolated cat intestine was determined. The oxygen consumption of such a preparation always remains constant. In perfusing experiments on hind limb preparations the oxygen uptake always increases markedly. This increase generally continues for the entire experimental period of two hours which is the time most often used in my experiments. The marked difference between the changes in oxygen uptake in a typical experiment on a liver preparation as compared with a hind limb preparation

is shown in Fig. 1. The oxygen uptake has been followed by frequent photoelectric determinations of the oxygen content in the venous blood. The galvanometer readings

in each experiment have been standardized by . 5 at least 4 determinations of the venous oxygen \subseteq content by the Van Slyke technique. Care has been taken to obtain, as great differences between the oxygen content in the samples used for the standardization as possible. The oxygen content in the arterial blood was determined with the VAN SLYKE technique at the beginning and at the end of the experimental period and in some experiments also in the middle of this period. Though the initial pronounced decrease in oxygen consumption is only observed in experiments with isolated livers it can not of course be ruled out that this decrease might be due to an impairment of the circulation in the liver or some other damage developing during the first

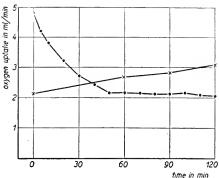


Fig. 1. Spontaneous changes in oxygen consumption during artificial perfusion of a cat liver (•——•) and a hind limb preparation (×——×).

period after the isolation of the organ. The question whether it is possible to restore the oxygen uptake after it has attained its low and rather constant level must be of decisive importance for the evaluation of the phenomenon.

On the assumption that the decrease in oxygen consumption is due to a disappearance of some substance present in fresh blood but gradually used up by the liver the simplest way to try to restore the oxygen uptake would be to renew the blood after the drop in oxygen uptake has developed. The result of such a simple experiment is shown in Fig. 2. As is seen the addition of fresh blood to the perfusion apparatus causes a marked but transitory increase in the oxygen uptake. A quantitative comparison between the increase obtained by adding fresh blood and the initial drop in oxygen uptake is difficult since it is not possible to renew the blood in the apparatus completely. It is only possible to remove some of the blood and add some fresh blood. In this way

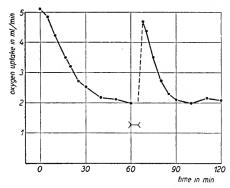


Fig. 2. Oxygen uptake of isolated cat liver, between > and < perfusion blood partly exchanged with fresh blood.

not more than about 50% renewal of the blood is obtained. As some change in the cell volume of the perfusion blood resulting from the addition of fresh blood cannot be avoided, and as this alters the standardization of the galvanometer readings care has been taken to draw simultaneously a sample of arterial and venous blood for Van Slyke determinations as near as possible to the "peak" as judged from the galvanometer readings. In this way the magnitude of the increase in the neighbourhood of the maximum is ascertained by the Van Slyke technique.

In some experiments blood used for the perfusion of a liver for one to one and a half hours has been used for perfusion of an other freshly

prepared liver. In these experiments the oxygen uptake of the second liver was low from the start of the perfusion and remained low.

From these simple observations it seems safe to conclude that the observed drop in oxygen uptake in an isolated liver is due to changes in the blood and not to changes in the liver tissue as such.

Though as mentioned the most probable assumption is that the decline in oxygen uptake is due to the disappearance of some substance from the blood the possibility remains that it is due to accumulation of some inhibitory substance. Also in that case addition of fresh blood might be expected to cause an increase by dilution of the inhibitory agent. Though the course of the fall in oxygen uptake appears incompatible with such an assumption an attempt has been made to elucidate this possibility experimentally.

Some livers were perfused with washed red, blood corpuscles suspended in an artificial plasma. Dextran, a polysaccharide preparation, was added to the artificial plasma to secure a normal colloid osmotic pressure. Though the result of these experiments was not quite clearcut due to technical difficulties which need not be mentioned here it can safely be stated that only a very slight initial fall in oxygen uptake was observed in

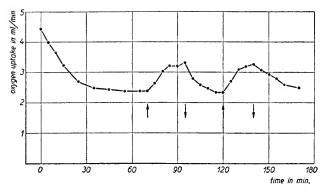


Fig. 3. Oxygen consumption of isolated cat liver. Hind limb preparation shunted in at ↑ and out at ↓.

these experiments.

The observations so far mentioned support the assumption that the liver normally is supplied by the blood with a substance which affects its metabolic rate.

That this hypothetic substance probably is not a specific hormone formed in one of the endocrine glands is indicated by experiments carried out in the following way.

A perfusion apparatus with a double pump and two circuits

but with a common oxygenator and blood reservoir was used. A liver was isolated and attached to one of the circuits, the other being short circuited. The oxygen uptake of the liver was followed in the usual way and when the oxygen uptake had dropped a hind limb preparation was attached to the previously short circuited circuit. The venous blood returning from the liver and the hind limb preparation in this way is mixed in the oxygenator and the blood reservoir and the liver is supplied with a mixture of blood returning from the liver and the hind limb preparation. As seen from Fig. 3 the oxygen uptake of the liver starts to increase as soon as the hind limb preparation is shunted in. In about 15 minutes it reaches a fairly constant level which is maintained until the hind limb preparation is shunted out. The shunting out of the hind limb preparation is followed by a gradual decline in the oxygen uptake following a course similar to that of the initial fall. The increase is marked though the initial high oxygen uptake is not restored. In the experiment presented in Fig. 3 the hind limb preparation after having been left without circulation for 35 minutes again was shunted in for 20 minutes. The response was practically identical with the first response. The correspondence between the two response must be emphasized inasmuch as it speaks strongly against the possibility that lactic acid may be responsible for the increase in oxygen uptake. This point will be discussed later; it may be only mentioned that the lactic acid concentration in

the blood at the start of the experiment (oxygen uptake 4.08 ml/min) was 20 mg%, at the maximum of the first response (oxygen uptake 3.30 ml/min) 7 mg% and at the maximum of the second response (oxygen uptake 3.25 ml/min) 26 mg%.

These observations on the rate of oxygen consumption in the isolated liver would probably not have been published if the effect of the periphery on the metabolic rate of the liver had not been revealed in a much more striking manner in some other experiments performed for quite a different purpose.

A cat was hepatectomized by connecting the protal vein with the right renal vein through a cannula of suitable shape and ligating the hepatic vessels. Heparin had been injected to prevent clotting. The blood sugar concentration of the animal was kept as constant as possible by continuous intravenons injection of glucose. In some experiments in which the hepatectomy was not successful the cat was eviscerated. No difference has been observed in the results obtained in experiments on hepatectomized and eviscerated animals. As soon as the operation was finished a cat liver was isolated and run with artificial perfusion for 35 to 50 minutes. After this period of time, the oxygen uptake of the liver has fallen to a constant low level. The glucose concentration in the perfusion blood was followed. From these determinations the glucose output of the isolated liver can be computed with fair accuracy as the blood volume is known. 35 to 50 minutes after the start of the artificial perfusion the oxygen uptake of the liver was determined by means of the VAN SLYKE technique.

The isolated liver was then connected with the hepatectomized cat in the following way. The venous outflow from the liver was connected with the jugular vein of the hepatectomized cat which henceforward shall be denoted the "donor". From the carotic artery of the donor, blood was allowed to run into a 100 ml cylinder containing about 50 ml of blood. Simultaneously the pump was shifted from the blood reservoir connected with the oxygenator to the 100 ml cylinder cutting out the oxygenator and reservoir from the circuit. The blood which flowed from the donor into the cylinder was then taken up by the pump and sent through the liver at a constant rate determined by the pump. From the liver the blood returned to the donor. By means of a clamp on the outflow from the carotic artery of the donor it was fairly easy to manage to keep the bloodvolume in the cylinder constant, *i.e.*, to secure that the amount of blood leaving equalled the amount of blood entering the donor.

When the liver was connected with the donor the glucose infusion was stopped. At suitable intervals samples were drawn simultaneously from the blood entering and leaving the liver. Oxygen, carbon dioxide, glucose, and lactic acid determinations have been performed on these samples. Oxygen and carbon dioxide were determined with the Van Slyke technique, glucose according to Hagedorn-Jensen, and lactic acid according to Barker and Summerson modified by LePage.

The results related to our problem are presented in Table I. It is seen that within 10 minutes after the connection of the liver with the donor the rate of oxygen consumption in the liver has increased 100% or even more. One hour after the connection the oxygen uptake of the liver in most experiments shows a slight further increase. In other words the connection with a donor of a liver run with artificial perfusion until the oxygen uptake has dropped to a low level increases the rate of oxygen uptake to a rate similar to that observed immediately after isolation of the liver, *i.e.*, presumably to the normal rate. It may be mentioned that this very considerable change in rate of oxidations is not accompanied by any change in the respiratory quotient.

TABLE I

OXYGEN CONSUMPTION OF CAT LIVERS BEFORE, 10-15 MINUTES AND 60 MINUTES AFTER CONNECTION

WITH A "DONOR" ml/min

| Before       | 10-15 min after | 60 min after |
|--------------|-----------------|--------------|
| 1.8          | 3.8             | 3.9          |
| 2.6          | 5.0             | 5.2          |
| 2.4          | 5.1             | 4.8          |
| 2.2          | 4.0             | 4.7          |
| 1.7          | 4.6             | 5.0          |
| 2,2          | 5-4             | 4.7          |
| 2.0          | 4.4             | 4.9          |
| Average 2.14 | 4.61            | 4.74         |

As the high rate of oxygen consumption in the liver after connection with the donor is maintained or even increases slightly during the entire experimental period though the liver is still artificially perfused the possibility that the decline in oxygen uptake might be a direct consequence of the artificial perfusion is ruled out. The conditions before and after connection with the donor differ in only one respect. Before the connection when the blood is oxygenated in the oxygenator the oxygen tension is higher in the blood entering the liver than after the connection when the blood is oxygenated in the lungs of the donor. Though it is most improbable that the oxygen tension of the blood entering the liver is of any significance a few experiments have been carried out in which the perfusion blood was oxygenated with alveolar air collected in a Douglas bag instead of the ordinary mixture of oxygen and 4% carbon dioxide. The oxygen uptake of the liver in these experiments showed exactly the same variations as in experiments carried out with the usual technique.

The glucose output from an isolated cat liver averages about 2 mg per minute. The glucose output from a liver after connection with a donor averages about 9 mg per minute. The extra amount of glucose given off by a liver after connection with a donor undoubtedly originates from lactic acid.

In ordinary perfusion experiments on cat livers the lactic acid concentration rapidly falls to very low levels (3 to 5 mg%). In experiments in which the artificially perfused liver is connected with a donor the lactic acid concentration in the blood with which the liver is supplied is as high as 30 to 50 mg%. A definite drop in lactic acid concentration from ingoing to outgoing blood corresponding roughly to the increase in glucose concentration is demonstrable.

As the lactic acid concentration declines during the first period of a liver perfusion experiment during which the oxygen uptake falls off also and as the lactic acid concentration is markedly increased after connection with a donor when the oxygen consumption increases strongly one might think that the concentration of lactic acid in the blood is responsible for the changes in the oxygen uptake of the liver. Observations have previously been mentioned however which do not agree with such an assumption. Furthermore a number of experiments have been carried out in which L (+) lactic acid was added to the perfusion blood. If at the start of the perfusion L (+) lactic acid is added to the blood in amounts increasing the concentration to well above 100 mg% the decline in oxygen uptake proceeds as usual and if lactic acid in varying amounts is

added to the blood after the oxygen uptake has reached its constant low level only a very slight increase in the oxygen uptake or no increase at all is observed. Consequently the possibility that the lactic acid concentration in the blood is responsible for the changes in oxygen consumption observed in these experiments can be definitely ruled out.

If lactic acid is added to the blood after the oxygen uptake of an isolated liver has been allowed to drop off the rate of disappearance of lactic acid amounts to only one fourth to one third of the rate observed in a liver connected with a donor. Thus not only the rate of oxygen consumption but also the rate of a reaction such as conversion of lactic acid to glucose or glycogen is influenced by the hypothetical substance present in fresh blood. The statement appears justified that this substance influences the "metabolic rate" of the liver.

The nature of the substance influencing the metabolic rate of the liver has not been elucidated; accordingly, this paper can be considered only as a preliminary note. A series of substances however can be ruled out since they have no effect on the rate of oxygen uptake in the liver when added to the blood about one hour after the start of the perfusion. Some of these substances have been added to the blood in a single dose, others have been added continuously at a rate giving concentrations in the blood comparable with the normal concentrations. Without going into details a few of the substances tested so far are listed (Table II).

#### TABLE II

"Kochsaft" of muscle
Fresh muscle extract
ATP
Creatine
Cytochrom C
Glutathione
Citric acid
Oxalo-acetic acid
Fumaric acid
Succinic acid
Pyruvic acid
Lactic acid
Acetic acid

Choline
Methionine
Tyrosine
Tryptophan
Arginine
Threonine
Ascorbic acid
Adrenaline
nor-Adrenaline
Desoxycorticosterone glycoside (Ciba)
"Corsunal"\*
Insulin
Fresh crude extract of anterior pituitary

Among the substances listed in Table II only adrenaline and nor-adrenaline had a definite but quite transitory effect of increasing oxygen uptake. This effect, however, could not be maintained by continuous addition of the substances.

It must be mentioned that pyruvic acid and the aminoacids glycine and alanine in large doses (300 mg) have a marked effect on the oxygen uptake in the isolated liver. As continuous addition of pyruvic acid at a rate of 2 mg per minute (blood flow 50 to 60 ml/min) has no effect on the oxygen uptake and as the amino acid content in blood perfused through a liver does not decrease as does the oxygen uptake during the first period of the experiment it appears that pyruvic acid and amino acids can safely be ruled out as factors responsible for the changes in oxygen uptake in the liver observed in these experiments.

The problem to which attention is directed in the present paper undoubtedly is References p. 329.

<sup>\*</sup> Extract of ox-adrenals prepared by Nordisk Insulin Laboratory according to Grollman and Firor

related to the observation made by many investigators3, 4, 5, 6, 7 that the respiration of tissue slices is higher and more stable in serum than in Ringer solution. Though this observation is not absolutely identical with those of the writer, it appears most probable that the substance (or substances) in serum which enchances tissue respiration is the same as the substance (or substances) which is gradually removed from the blood by an isolated liver causing a decline in the rate of oxidations. The question of the nature of the serum constituents which enchance tissue respiration has been delt with in a rather explicit manner by Warren in two publications. In the first of these8 it has been demonstrated that the stimulating effect of serum on tissue respiration partly can be attributed to its bicarbonate content. According to Warren the maximal effect of adding bicarbonate to a Ringer-phosphate medium is obtained at a concentration of only 3 mM per liter. Variations in the bicarbonate concentration at higher levels are without any influence on the rate of oxidations. Since whole blood under constant and fairly high carbon dioxide pressure has been used in the experiments described one can certainly rule out changes in bicarbonate content as being responsible for the observed changes in oxygen uptake in the isolated liver.

In accordance with Canzanelli et al.³, Warren finds substances capable of enchancing the respiration of tissue slices in the ultrafiltrate of serum. Only about 50% of the effect can be attributed to bicarbonate. In his second paper Warren³ reports attempts to fractionate serum with respect to its action in enchancing tissue respiration. From his elaborate experiments Warren concludes that lactic acid and amino acids are not involved in the stimulating effect of serum on tissue respiration. I draw the same conclusion from my observations. Warren further suggests that the active substance is a dicarboxylic acid, but he has not put this assumption on a direct trial by adding dicarboxylic acids to the Ringer-phosphate medium used in his experiments. In my experiments I have tested different organic acids assumed to be formed as intermediates in tissue metabolism. However, no effect on the low oxygen uptake of the isolated liver was observed.

#### SUMMARY

Observations are presented indicating that the normal metabolic rate of the liver is dependent on a substance (or substances) formed in the extrahepatic tissues and carried to the liver through the blood. This still unidentified substance is used or destroyed in the liver tissue.

#### RÉSUMÉ

L'auteur présente des observations indiquant que la vitesse normale du métabolisme du foie dépend d'une substance (ou de substances) formée dans les tissus extrahépatiques et qui est amenée au foie par le sang. Cette substance non encore identifiée est utilisée ou détruite dans le tissu hépatique.

#### ZUSAMMENFASSUNG

Beobachtungen werden beschrieben die darauf hinweisen, dass die Normalgeschwindigkeit des Lebermetabolismus von einer Substanz (oder von Substanzen) abhängt, die in ausserhalb der Leber gelegenen Geweben gebildet und durch das Blut der Leber zugeführt wird. Diese noch nicht identifizierte Substanz wird im Lebergewebe verbraucht oder zerstört.

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# IS ACETALDEHYDE AN INTERMEDIARY PRODUCT IN NORMAL METABOLISM?

by

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Mainly through the work of MEYERHOF, PARNAS, EMBDEN, and CORI, their collaborators and pupils, the intermediary products of the first part of carbohydrate metabolism are well known. The intermediary products have been isolated and the enzymes involved thoroughly studied. It is now generally accepted that glycogen or glucose is broken down to pyruvate through a series of phosphorylated compounds. Pyruvate forms a "natural dividing point" between the anaerobic and the aerobic phases of carbohydrate metabolism. It has, however, been extremely difficult to study the intermediary products and the corresponding enzymes in volved in the further oxidation of this substance. Several hypotheses concerning this part of carbohydrate metabolism have been proposed. The experimental facts hitherto obtained seem to be best explained by Krebs' citric acid cycle-theory. The individual processes are well known and need no further description (KREBS, 1943). Nevertheless it is not known whether other processes are also involved in the oxidation of pyruvate and alternative schemes have been proposed. The early theory of Thunberg (1920) and Knoop (1923) suggests that pyruvic acid is decarboxylated to acetaldehyde which is then oxidized to acetic acid. This compound is in turn condensed to succinic acid. Their theory has now been abandoned, mainly because it has been impossible to demonstrate any formation of succinic acid from acetic acid in living cells or cell extracts. It has, however, been shown by several authors that acetaldehyde can be formed during tissue metabolism. In in vitro experiments with minced tissues acetaldehyde has been trapped by means of aldehyde fixatures following the technique of Neuberg. Hirsch (1923) identified acetaldehyde formed in muscles of frogs or fishes. Neuberg and Gottschalk (1924) showed the formation of acetaldehyde in different tissues of warm-blooded animals and their results have been confirmed and enlarged by Palladin and Utewski (1929), Gorr (1932), TANKÓ, MUNK, and ABONYI (1940) and others. Addition of pyruvate to the minced muscles increases the yield of acetaldehyde (UTEWSKI, 1929) and the formation of acetoin, a condensation product of acetaldehyde and pyruvic acid, from pyruvate has been shown by Green et al. (1941) and by Stotz, Westerfeld, and Berg (1944). In animal tissues acetate was identified as an oxidation product of pyruvic acid by Krebs AND JOHNSON (1937), WEIL-MALHERBE (1937) and LONG (1938). It was shown that pyruvate anaerobically dismutes into lactate + acetate + carbon dioxide. Although Krebs and Johnson emphasize that this process in animal tissues differs from that of decarboxylation of pyruvic acid in microorganisms, it cannot be excluded with certainty that acetaldehyde even in this process acts as an intermediary product.

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Acetaldehyde is oxidized very rapidly *in vivo* (Lubin and Westerfeld, 1945): and the acetoin formed *in vivo* also appears to be very rapidly metabolized.

Even if the citric acid cycle is the main path of the normal metabolism of pyruvic acid and some of the results showing a possible formation of acetaldehyde from pyruvic acid are due to artefacts in the sense that the biochemical processes only occur under more or less abnormal conditions, it is still possible that pyruvate in normal metabolism is partly broken down with acetaldehyde serving as an intermediary product. Hitherto no means have been available to decide to what extent this secondary path plays a rôle in the normal metabolic processes of the organism.

At the present experiments performed in this laboratory are able to throw a light on the question.

Hald, Jacobsen, and Larsen (1948) have shown that individuals given tetraethylthiuramdisulphide (Antabuse) will give a series of symptoms after ingestion of minute amounts of alcohol. The occurrence of these symptoms is due to an increased formation of acetaldehyde from alcohol, resulting in an increased concentration of acetaldehyde in the blood (Hald and Jacobsen, 1948; Asmussen, Hald, and Larsen, 1948; and Larsen, 1948). If the metabolic rate of acetaldehyde is slowed after ingestion of Antabuse, the increased concentration of this substance in the organism is easily explained. Preliminary experiments in this laboratory showed, however, that no difference in the rate of acetaldehyde elimination in normal and Antabuse-treated animals could be seen when acetaldehyde was given during short periods and in such an amount that the final concentration of acetaldehyde in the blood was 20–25 mg/%. In collaboration with Dr.'s Jens Hald and Valdemar Larsen I have made a series of further experiments showing that the metabolic rate of small concentrations of acetaldehyde is decreased in animals treated with Antabuse. These experiments will be published in detail by Hald, Jacobsen, and Larsen.

A series of rabbits weighing from 2.0–2.5 kg were given 0.50 g Antabuse 48, 24 and 16 hours prior to the experiment. The animals were anesthetized with urethan. Blood samples were taken from a cannula inserted in the carotid artery. Coagulation was prevented by the injection of 1500 units of heparin intravenously. Acetaldehyde determinations were made by Stotz's method. A cannula was inserted into the jugular vein. Two to ten per cent solutions of acetaldehyde in Tyrode's solution were infused through the cannula at a known constant rate. The infusing apparatus consisted of a 10–30 ml syringe, the piston of which was controlled by a screw driven mechanically by a gramophone motor. The experiments generally lasted 1½–2½ hours. During this period the infusion rate was maintained at a constant level which did not exceed the capacity of the rabbits to metabolize acetaldehyde. There was no accumulation of acetaldehyde in the tissues during the experiment.

An average sized rabbit is usually capable of eliminating 7–8 mg acetaldehyde per minute. The concentration of acetaldehyde in the blood was determined 30 minutes after the beginning of the infusion and at intervals of  $^{1}/_{2}$ – $^{3}/_{4}$  hours. The levels of acetaldehyde in blood corresponding to a fixed infusion rate of acetaldehyde varying between 0.75 mg and 9 mg per minute were determined in two series of rabbits: one normal series, and one consisting of rabbits treated with Antabuse in the manner described above. A considerable variation of the blood acetaldehyde is noted from time to time although the infusion rate was kept as constant as possible. The results of the experiments are tabulated in Fig. 1. A clear difference between the concentration of acetaldehyde in

blood in the two series is shown. When the same amount of acetaldehyde is metabolized, the level of acetaldehyde in blood is higher in the Antabuse-treated animals than in the untreated ones. The smaller the amounts of acetaldehyde metabolized per minute, the greater is the relative difference between the two groups. When 0.75–2.0 mg is infused per minute, the acetaldehyde level in blood of the Antabuse-treated rabbits is 5–10 times that of the normal animals, whereas it is less than twice when 8–9 mg are infused per minute. The same results are obtained in perfusion experiments with isolated liver and hind limbs. An account of these experiments will be published at a later date.

If acetaldehyde is found as a normal split product in metabolism, the experiments described here show that this will result in an increased concentration of acetaldehyde in the blood of rabbits treated with Antabuse. Acetaldehyde in blood was determined in normal and Antabuse-treated rabbits. The results are given in Table I. No significant statistical difference between the two groups is seen.

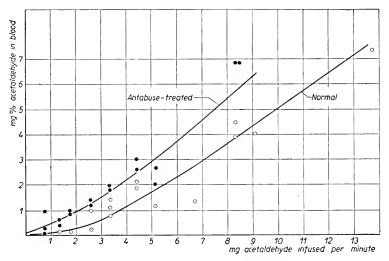


Fig. 1. Correlation between infusion rate of acetaldehyde into the jugular vein and mg acetaldehyde per 100 ml blood in normal rabbits and rabbits treated with Antabuse (tetraethylthiuramdisulphide)

Similar results are obtained in perfusion experiments. A series of livers and hind limbs from normal rabbits and rabbits treated with Antabuse were artificially perfused with blood as described by Nielsen (1933). On an average the livers weighed about 80 g, and the muscles of the hind limbs 430 g. The average oxygen uptake per minute was 1–3 ml per minute in the livers and 2–4 ml per minute in the hind limbs. When acetaldehyde was added to the perfusion blood, the blood which passed through the livers or muscles from Antabuse-treated animals showed a considerably higher concentration of acetaldehyde than blood that passed through organs of normal animals. From the amount of blood perfused per minute and the difference in acetaldehyde concentrations in the blood before and after the perfusion it is possible to calculate the amount of acetaldehyde passing into the perfusion blood per minute. If any substantial quantity of acetaldehyde is formed during normal metabolism, a difference should be seen between the perfusion experiments made with normal animals and with Antabuse-treated animals. As seen in Table II this is not the case. At times the acetaldehyde

TABLE I

|                                                       | Antabuse treated rabbits | Normal rabbits     |
|-------------------------------------------------------|--------------------------|--------------------|
| Number of animals                                     | 28                       | 19                 |
| Range of acetaldehyde concentration in blood          | o.o1 to 0.25 mg %        | o.oo to o.30 mg %  |
| Average and standard deviation of average             | 0.104 ± 0.012 mg %       | 0.085 ± 0.017 mg % |
| $\sigma=$ Standard deviation of single determinations | 0.021 mg %               | 0.023 mg %         |

Acetaldehyde in mg 100 ml blood in rabbits treated with Antabuse (tetraethylthiuramdisulphide) and in normal rabbits

TABLE II

|                                                       | Antabuse<br>treated rabbits | Normal rabbits   |            |
|-------------------------------------------------------|-----------------------------|------------------|------------|
| Number of experiments                                 | 16                          | 17               |            |
| Range of mg acetaldehyde formed per minute            | -0.04  to + 0.15            | - 0.02 ± 0.11    | liver      |
| Average and standard deviation of average             | 0.032 ± 0.013               | o.o3o ± o.o11    |            |
| $\sigma=$ Standard deviation of single determinations | 0.053                       | 0.047            | ]          |
| Number of experiments                                 | 9                           | 12               |            |
| Range of mg acetaldehyde formed per minute            | -0.02 to +0.12              | -0.07  to + 0.07 | hind limbs |
| Average and standard deviation of average             | 0.012 ± 0.006               | 0.008 ± 0.001    | I ma mios  |
| $\sigma=$ Standard deviation of single determinations | 0.017                       | 0.030            |            |

Acetaldehyde formation per minute in isolated organs from rabbits treated with Antabuse and from normal rabbits

formation is negative. This indicates that the concentration of acetaldehyde is lower in the blood which has been perfused through the organ than in the blood which enters the organ. Of course the analytical error is rather high when determining small concentrations of acetaldehyde and so will influence the results considerably. Furthermore substances other than acetaldehyde may give reactions which influence the determinations to a considerable degree when small concentrations of acetaldehyde are found in the blood. Nevertheless the production of acetaldehyde under the above mentioned conditions appears to be of very little importance.

Thus it may be concluded that very little, if any, acetaldehyde can be formed during normal metabolism and that the alternative paths in metabolism in which acetaldehyde is supposed to be an intermediary product, do not play a significant rôle.

#### SUMMARY

It has been shown that acetaldehyde metabolism is delayed in animals treated with tetraethylthiuramdisulphide (Antabuse).

No increase of acetaldehyde formation can be seen in total organisms and in isolated livers and muscles from rabbits treated with Antabuse.

From these observations it is concluded that acetaldehyde plays a very insignificant rôle as an intermediary product in normal metabolic processes.

#### RÉSUMÉ

On montre que le métabolisme de l'acetaldehyde est retardé dans les animaux traités au tetraéthylthiuramdisulfide (Antabuse).

Aucune augmentation de la formation d'acetaldehyde n'a pu être observée dans les organismes entiers et dans les foies et les muscles de lapins traités à l'Antabuse.

De ces observations nous concluons que l'acetaldehyde joue un rôle très peu important dans les processus métaboliques normaux.

#### ZUSAMMENFASSUNG

Es wird gezeigt, dass der Metabolismus des Acetaldehyds in mit Tetraäthylthiuramdisulfid (Antabuse) behandelten Tieren verzögert ist.

Eine Zunahme der Acetaldehydbildung in ganzen Organismen oder in isolierten Lebern und Muskeln von mit "Antabuse" behandelten Kaninchen wurde nicht beobachtet.

Aus diesen Beobachtungen wird geschlossen, dass das Acetaldehyd eine sehr unbedeutende Rolle als Zwischenprodukt der normalen metabolischen Prozesse spielt.

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### THE QUANTUM EFFICIENCY OF PHOTOSYNTHESIS

by

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Photosynthesis is a unique endothermic photochemical reaction in which chemical energy is gained from visible light energy by the combined action of several quanta. Nothing similar is known in the nonliving world. It was first reported a quarter of a century ago¹ that in photosynthesis the greater part of the absorbed visible light energy could be converted into chemical energy under optimum conditions. Indeed, no more than four quanta of red light seemed to be necessary to produce one molecule of oxygen gas, which is close to the thermodynamic requirement of three quanta. It is easy to understand that this result, lacking any analogy, has sometimes been doubted by theoreticians, and it is a fact that certain investigators have raised methodological objections². For this reason we have reinvestigated the question of the minimum quantum requirement of photosynthesis as measured by oxygen and carbon dioxide gas exchange. The present paper is a short summary of our findings by new and simplified methods.

#### I. CULTIVATION OF CELLS

A strain of Chlorella pyrenoidosa, isolated in New England and identified by Dr. Florence Meier of the Smithsonian Institution, and for many years in laboratory use, was cultivated in tall Drechsel gas washing bottles containing 200 ml of the following salt solution: 5 g MgSO<sub>4</sub>·7 H<sub>2</sub>O, 2.5 g KNO<sub>3</sub>, 2.5 g KH<sub>2</sub>PO<sub>4</sub>, 2 g NaCl, and 5 mg FeSO<sub>4</sub>·7 H<sub>2</sub>O, in 1 liter of filtered, unsterilized well water (p<sub>H</sub> 4.5-5). The cultures were maintained at a room temperature of 25-30° C, and were aerated with 5% CO<sub>2</sub> in air at a rate (~500 ml per minute) rapid enough to prevent cell settling, and were constantly illuminated with a 100-watt incandescent lamp at a distance of about 30 cm. Cells cultivated by this method gave more uniform material and more regular manometric results than when cultivated by the older method (1, p. 427) in which slowly aerated cells settled down in Erlenmeyer-shaped flasks and became partially anaerobic until reshaken up, and in which lowered light intensities were employed for the terminal cultivation phase.

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The cultures were used for the experiments in the present work after 2–10 days growth, when they contained 200–1000  $\mu$ l cells, depending upon the amount of initial inoculation. Usually 50–100  $\mu$ l cells per 200 ml medium were employed as inoculum, grown as just indicated. Bacterial growth during either cell culturing or manometric experiments was found with a haemocytometer to be negligible, due to the low  $p_H$ , the lack of added organic matter in the synthetic medium, and possible antibiotics produced by the *Chlorella*.

The cells for experimental use were centrifuged in an International No. 2 Centrifuge at the lowest possible speed giving nearly complete settling in 10 minutes and were taken up, with or without further washing, in fresh nutrient medium at a concentration of 30–50  $\mu$ l cells per ml.

#### II. MONOCHROMATOR

A Steinheil glass 3-prism spectrograph operated with a focal length of 195 mm at F 3.5 for the collimator and a focal length of 710 mm for the telescope was used as a monochromator. The slit was illuminated with a 750-watt projection lamp. The image of the coiled filament at about  $20^{\circ}$  to its plane was projected onto the slit with an auxiliary lens. A 1000-watt voltage regulator was used to supply power to the lamp which operated at constant current.

The width of the entrance slit was about 2 mm, corresponding to about 20 m $\mu$  in the red. A slit was placed in the focal plane of the telescope and was adjusted to have a width of about 30 m $\mu$  covering the region 630 to 660 m $\mu$ . A lens was placed behind this slit to throw, in a weakly convergent beam, an image of the exit prism face on the bottom of the manometer vessel.

The area of the beam at the vessel was about 3 cm<sup>2</sup> and the energy flux was about 0.6 micro einsteins/min. This intensity was decreased when desired by placing in the light beam, just before the exit slit, blackened wire screens calibrated by the National Bureau of Standards.

#### III. MEASUREMENT OF LIGHT ENERGY

The energy of the light beam was measured by the recently developed chemical actinometer<sup>3</sup> whereby for each quantum of visible light absorbed one molecule of  $O_2$  is consumed. In the same or similar rectangular vessel as used for the yield determinations were placed 2 mg ethyl chlorophyllide, 200 mg thiourea, 7 ml pyridine, and  $O_2$  gas. The actinometer vessel was shaken in the thermostat at 20° C in the same manner and in the same cross-section of the light beam as the vessels with the cell suspensions were shaken during the yield determination. The total intensity of light, absorbed by the actinometer, should not exceed 0.3 microeinsteins per minute under our working conditions. Higher intensities, as used for the yield determinations, were diminished for this purpose by the calibrated screens. Several 10 minute periods were observed for every actinometer determination. When in t minutes the pressure change in the actino-

meter vessel is  $h_{O_2}$  mm, the total energy flux in the light beam in t minutes is  $\frac{h_{O_2} \cdot k_{O_2}}{22.4}$  or  $\frac{x_{O_2}}{22.4}$  microeinsteins (micromole quanta), where the vessel constant  $k_{O_2}$  is expressed

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in mm<sup>2</sup>. Then, when the oxygen developed by illuminating the green algae is  $n \mu l$  and the oxygen absorbed in the actinometer for the same time and beam of light is  $n' \mu l$ , the quantum requirement per mol of  $O_2$  developed in photosynthesis is simply 1 q = n' n.

#### IV. COMMENTS ON THE 2-VESSEL MANOMETRIC METHOD

If the yield q and the assimilatory quotient,  $\gamma = \frac{-CO_2}{+O_2}$ , are to be determined simultaneously, two vessels must be employed. If H be the pressure change in vessel I and H' that in vessel II, the  $x_{O_2}$  and  $x_{CO_2}$  values can be calculated by well known equations (see <sup>1</sup> and section 8).

The 2-vessel method, simple when the gas-exchanges in the dark are determined, requires special attention when applied to illuminated cells. As will be shown later, the illumination of the cells is an illumination with intermittent light. This intermittency should be equal in the two vessels, and this is attainable if the liquid volumes are equal in both vessels. Furthermore, the respiration in most cell suspensions gradually changes with time, so that the pressure changes in light will also change with time. Thus the two vessels should be darkened and illuminated simultaneously so that the conditions of the aforementioned equations are fulfilled, namely

$$X_{O_2} = X'_{O_2}$$
  
 $X_{CO_2} = X'_{CO_2}$ 

where the primed magnitudes refer to one vessel and the non-primed to the other.

These conditions may be satisfactorily met by the method of alternately shifting the mirror under the two vessels at periods of, c.g., 10 minutes, as indicated in Fig. 1, and discussed in the next section. After two or more cycles, the pressure readings for each vessel for light and dark periods may be averaged and the light action calculated from the differences between the pressure changes in light and dark. A possible error involving noncomparability of time periods is thus eliminated. This error has been one of the main sources of difficulty in *Chlorella*-photosynthesis experiments with the 2-vessel method.

#### V. PROCEDURE

Simple Haldane-Barcroft constant-volume manometers with small capillaries (0.8 mm diameter) with rectangular vessels attached were shaken horizontally (not by arc motion) at 140–180 (usually 150) cycles per minute at an amplitude of 2.0 cm in a water bath at 20° C. The two rectangular vessels of about 2.2 · 3.8 µl inside width and length and 13–14 and 18–19 ml volume respectively, were filled with 200–400 µl cells in 7 ml, thus the liquid volumes were identical and the gas spaces differed. The vessels (with capillary sidearm vents) were gassed on the bath, simultaneously with aid of a manifold, and with shaking. The horizontal (not arc) shaking was so effective that physical after-effects of gas equilibration in the transition periods of dark to light and vice versa were not appreciable even when the illumination produced photosynthesis far above the compensation point and pressure changes of 5–10 mm per minute were involved. The manometers were usually read without stopping. The end of the mano-

meter male joint was not flat rough but concave and polished, so that bubble formation in the capillary did not occur; nor did foaming.

As indicated in Fig. 1 a beam of red light (630-660 mu) of about 3-4 cm<sup>2</sup> area, produced by means of the Steinheil monochromator, entered the side of the thermostat

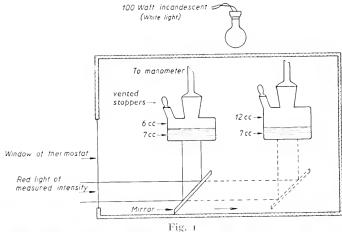




Fig. 2

through a two walled window and was reflected by a mirror onto the bottom of a vessel, alternately in the one or the other by either shifting the mirror or the manometers, depending on the design of the experiment. The red light entering the vessel was completely absorbed. To accomplish this, the amount of cells must be sufficiently great. The amount depends upon the chlorophyll content of the cells. It was found safe, to avoid loss of light, to have 300  $\mu$ l of cells in each vessel. No influence of the cell concentration on the yield was observed when light absorption was complete and shaking adequate. By this method, both  $O_2$  and  $CO_2$  exchanges were obtained simultaneously and independently for any and every desired period of measurement, and every yield determination was connected with an experimental determination of the relationship  $CO_2/O_2$ , so that earlier uncertainties concerning this ratio  $(\gamma)$  were eliminated.

#### VI. INTERMITTENCY OF ILLUMINATION

The cross-section of the light beam entering the vessels was about 3 cm<sup>2</sup>, that is, 3/8 of the bottom area of the vessel. It can be calculated, if we disregard the scattering of light, that the major part of the red light (75%) is absorbed within a distance of about 1 mm from the bottom of the vessel. This means that the light absorbing volume is only about 1/20 of the 7 ml of the cell-suspension.

Let now the intensity of the red light be so strong, that the oxygen consumption of the whole cell suspension is compensated by the oxygen evolution (compensation point for  $O_2$ ). Then the oxygen development in the absorbing volume of the cell suspension may approach 20 times the point where the cells become saturated with light and the increment yield zero (with our cell conditions the saturation intensity is about 30–40 times the compensation intensity). But we obtain maximum or high yields when the vessels are shaken as described at not only compensating but even considerably higher intensities, when the latter are provided by white light. This proves that under our shaking conditions the cells alternate so frequently between darkness and illumination that the concentrations of the participants of all dark reactions virtually retain their dark values — a consideration which shows the methodological importance of the kind and rate of shaking.

#### VII. YIELD DETERMINATIONS ABOVE THE COMPENSATION POINT

A limiting feature of most earlier yield determinations was the low total light intensity, so low that only a fraction of the respiration was compensated for by the light action. Thus the yield determinations were in a sense determinations of inhibited or diminished respiration. We have changed this situation by illuminating the vessels from above the thermostat by a 100-watt constant-voltage incandescent lamp (as diagrammed in Fig. 1), at such a distance that the pressure changes in the vessels become zero or positive; yield determinations were then made with measured amounts of red light added in the usual manner from below the vessel. The intensity of the white light at the vessel surface was considerably smaller per unit area than that of the red light but covered a many fold greater area and hence provided much more total effective light than did the red beam. Owing to this relationship of intensities it was possible to eliminate respiration as an experimental quantity, and to start the yield experiments at positive rather than negative pressures, and yet still obtain (as experience showed)

virtually as good yields from the red light, whether the base line were darkness or the white light.

Another limiting feature of the earlier yield experiments was the short duration of not only the periods of illumination (10 minutes) but also the total length of the experiment (commonly less than one hour). By the use of white light we have now succeeded in extending the duration of the manometric yield experiments up to at least 10 hours, if not indefinitely. The effects of this important advance are several. In general, the yields may now be determined under nearly the same conditions as obtain during the growth and cultivation of the cells, since the light intensity, temperature, medium, and gas phase during the growth and manometry are essentially the same, and furthermore we have found that the shaking does not change the cells under these conditions.

#### VIII. EXAMPLES OF DATA

Protocols 1, 2, and 3 provide examples of the data obtained.

#### PROTOCOL No. 1

Experiment of V-26-49. 20° C. 630–660 m $\mu$ . 5% CO<sub>2</sub> in air. 260  $\mu$ l of cells per vessel. Each vessel alternating 10′ in dark and 10′ red light; thus when vessel No. 5 was dark, vessel No. 3 was illuminated, and vice versa.

$$(\textit{Equation 1}) \text{ Action of light in 8o'} \ x_{O_2} = \frac{\frac{\text{H} \cdot \text{k}_{CO_2} - \text{H}' \cdot \text{k}'_{O_2}}{\text{k}_{CO_2}}}{\frac{\text{k}'_{CO_2}}{\text{k}'_{O_2}}} = + \text{151 } \mu \text{l}$$

(Equation 2) 
$$x_{CO_2} = \frac{H \cdot k_{O_2} - H' \cdot k_{CO_2}}{\frac{k_{O_2}}{k_{CO_2}} - \frac{k'_{O_2}}{k'_{CO_2}}} = -168 \,\mu\text{l}$$

Actinometer: -8.83 µl O2 per minute

Quantum efficiency for O<sub>2</sub>, 
$$\frac{1}{\varphi} = \frac{80 \cdot 8.83}{151} = \frac{4.7}{1}$$

Quantum efficiency for CO2, 
$$\frac{1}{\varphi} = \frac{80 \cdot 8.83}{168} = \underline{4.2}$$

Assimilatory quotient, 
$$\gamma = \frac{\text{CO}_2}{\text{O}_2} = \frac{-168}{+151} = -1.11$$

If  $\gamma = \frac{CO_2}{O_2} = -1.11$  is determined for a given cell suspension, then  $x_{O_2}$  and  $x_{CO_2}$  can be obtained by the pressure changes in light and dark in each single vessel. For example, in vessel No. 5, the following figures, taken immediately prior to the readings above, were obtained upon illumination with light of an actinometer value of  $-5.07~\mu l$   $O_2$  per minute:

$$(\textit{Equation 3}) \ \, \text{Action of light in 20}; \ \, x_{O_2} = H' \frac{k'_{CO_2} \cdot k'_{O_2}}{k'_{CO_2} + \gamma \cdot k'_{O_2}} = 20.2 \cdot 1.62 = + 32.8 \\ x_{CO_2} = -1.11 \cdot x_{O_2} = + 32.8 \cdot -1.11 = -36.4 \\ \text{Quantum efficiency for O}_2, \frac{1}{\varphi} = \frac{20 \cdot 5.07}{32.8} = \underline{3.1} \\ \text{Quantum efficiency for CO}_2, \frac{1}{\varphi} = \frac{20 \cdot 5.07}{36.4} = \underline{2.8}$$

#### PROTOCOL No. 2

Experiment of V-30-49.  $20^{\circ}$  C. 630-660 m $\mu$ . 5% CO<sub>2</sub> in air. 270  $\mu$ l of cells per vessel. Experiment I. Alternately dark and light each 10'. Actinometer for the red light (total) 5.4  $\mu$ l O<sub>2</sub> per minute. When vessel No. 5 was dark, No. 3 was illuminated and vice versa.

No. 5

Constants as in Protocol I

10' dark — 10.5 mm 10' light + 0.5 mm
10' ,, — 10.0 ,, 10' ,, 0 ,, 10' ,, — 3.5 ,, 10' ,, + 2.0 ,, 10' ,, — 9.0 ,, 10' ,, + 1.5 ,, 10' ,, — 2.5 ,, 10' ,, + 3.5 ,, 10' ,, — 9.0 ,, 10' ,, + 1.0 ,, 10' ,, — 8.0 ,, 10' ,, + 1.0 ,, 10' ,, — 8.0 ,, 10' ,, + 1.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.5 mm

60' dark — 55.0 mm 60' light + 4.0 mm
60': H' = 
$$4 + 55 = +59$$
 mm

No. 3

Constants as in Protocol I

10' dark — 2.0 mm 10' light + 3.0 mm
10' ,, — 3.5 ,, 10' ,, + 2.0 ,, 10' ,, + 3.5 ,, 10' ,, — 2.5 ,, 10' ,, + 3.5 ,, 10' ,, — 0 ,, 10' ,, + 3.0 ,, 10' ,, — 1.0 ,, 10' ,, — 5.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.0 ,, 10' ,, + 5.0 ,, 10' ,, — 1.0 ,, 10' ,, — 1.5 mm 60' light + 20.0 mm
60': H' =  $4 + 55 = +59$  mm

Experiment II: Both vessels were now constantly illuminated with a 100-watt incandescent lamp of nonmeasured \* light intensity and red light of measured intensity added for alternating periods of 5'. Actinometer for the red light (total) 5.4  $\mu$ l  $O_2$  per minute.

No. 5

No. 3

5' white 
$$+$$
 18.5 mm 5' white  $+$  red  $+$  22.0 mm 5' white  $+$  14.0 mm 5' white  $+$  red  $+$  15.0 mm

5' ,,  $+$  18.0 ,, 5' ,,  $+$  22.5 ,, 5' ,,  $+$  14.0 ,, 5' ,,  $+$  16.5 ,,

5' ,,  $+$  16.5 ,, 5' ,,  $+$  22.0 ,, 5' ,,  $+$  12.5 ,, 5' ,,  $+$  16.5 ,,

5' ,,  $+$  17.5 ,, 5' ,,  $+$  20.5 ,, 5' ,,  $+$  14.0 ,, 5' ,,  $+$  14.0 ,,

5' ,,  $+$  17.0 ,, 5' ,,  $+$  23.0 ,, 5' ,,  $+$  11.5 ,, 5' ,,  $+$  15.0 ,,

25' white  $+$  87.5 mm 25' white  $+$  red  $+$  110 mm

30' white  $+$  78 mm 30' white  $+$  red  $+$  91.5 mm

30': H = 91.5  $-$  78 =  $+$  13.5 mm

25': H' = 110  $-$  87.5 =  $+$  22.5 mm

Calculation of quantum efficiency for experiment I (Dark ± Red)

In 60': H + 31.5 mm H' + 59 mm

Applying equations (1) and (2), protocol (1)

In 60' 
$$x_{O_{2}}^{1} = +70.4 \ \mu l$$
  
 $x_{CO_{2}} = -56.0 \ \mu l$   $\gamma = \frac{CO_{2}}{O_{2}} = -0.8$   
Quantum efficiency for  $O_{2}$ ,  $\frac{1}{\varphi} = \frac{60 \cdot 5.4}{70 \cdot 4} = \underline{4.6}$   
Quantum efficiency for  $CO_{2}$ ,  $\frac{1}{\varphi} = \frac{60 \cdot 5.4}{50} = \underline{5.8}$ 

Calculation of quantum efficiency for experiment II (White ± Red)

In 25': H + 11.3 mm H' + 22.5 mm

Applying equations (1) and (2), protocol (1)

In 25' 
$$x_{O_2} = +30.3 \ \mu l$$
  
 $x_{CO_2} = -27.2 \ \mu l$   $\gamma = \frac{CO_2}{O_2} = -0.90$   
Quantum efficiency for  $O_2$ ,  $\frac{1}{r} = \frac{25 \cdot 5.4}{30.3} = 4.5$   
Quantum efficiency for  $CO_2$ ,  $\frac{1}{r} = \frac{25 \cdot 5.4}{27} = 5.0$ 

<sup>\*</sup> But kept constant by a 500-watt voltage regulator.

Experiment III, with the same cells, was performed between experiments I and II, the white light being, however, of somewhat lower intensity. Here only one vessel (No. 5) was used; but if we take as y the average value of experiments I and II, that is -0.85, x<sub>02</sub> can be calculated according to equation (3), protocol (1). The readings in vessel (5) were:

$$25'$$
: H' =  $58.5 - 30.5 = +28 \text{ mm}$ 

and with  $\gamma = -0.85$ 

$$_{25}' \, \mathrm{x}_{\mathrm{O}_{2}} = + \, 34 \, \mu \mathrm{l}$$

The quantum efficiency with the actinometer value of experiments I and II (5.4 \(mu\)I O2 per minute) was

$$\frac{1}{\varphi} = \frac{25 \cdot 5.4}{34} = 4.0 \text{ for O}_2$$

The total duration of these experiments was 7 hours from the time of initial equilibration until the last yield determination that gave a value  $\frac{1}{\omega} = 4.5$  for oxygen, which was obtained at approximately 4 times the compensation point. The final pH in the cell suspensions was 5.4.

#### PROTOCOL No. 3

Comparison of the yield in carbonate-bicarbonate mixtures and in culture medium

Experiment of VI-1-49. 20° C. 630-660 mμ. Three vessels, in each 7 ml cell suspension, containing 200 µl of cells. Cultures centrifuged, then washed once in, and taken up in, carbonate-bicarbonate mixture. Intensity 5.4  $\mu$ l O<sub>2</sub> per minute.

I. Vessel No. 7.

 $\begin{array}{lll} V & = 13.824 \ ml \\ v_f & = 7.00 \ ml \\ k_{O_2} = 0.657 \end{array}$ 

Gas space air. Solution 85 parts M/10 NaHCO<sub>3</sub> + 15 parts M/10 K<sub>2</sub>CO<sub>3</sub>; p<sub>H</sub> 9.2. At compensation point with white light.

15' white light 0
15' ,, , + red light + 11.5 mm
15' ,, , , + o
15' ,, , + m + m + 11.5 ,,
15' ,, , + m + m + 11.5 ,,
15' ,, , - 0.5 ,,
Light action 
$$30' + 23 + 0.5 = + 23.5$$
 mm = 15.4  $\mu$ l
$$\frac{1}{\varphi} = \frac{30 \cdot 5.4}{15.4} = \frac{162}{15.4} = \underline{10.5}$$

II. Vessels Nos. 3 and 5, containing 7 ml culture medium,  $p_{H}$  4.9, with 200  $\mu l$  of cells each. Cultures centrifuged, then washed once in, and taken up in, fresh culture medium. Gas space 5% CO<sub>2</sub> in air. Mirror shifted every 10' from one vessel to the other; actinometer 5.4  $\mu$ l O2 per minute for red light.

$$x_{O_2} = +41.3 \text{ mm} \atop x_{CO_2} = -43.0 \text{ ,,}$$
  $\gamma = -1.04$   
 $\frac{1}{\varphi} = \frac{30.5.4}{41.3} = \frac{162}{41.3} = 3.9$ 

III, Vessel No. 7, with same cells as before but without white light (below compensation-point). PH 9.2

Light action 20'

$$62.7 - 46 = + 16.7 \text{ mm} = 11 \mu l$$

$$\frac{1}{m} = \frac{20.5.4}{11} = 9.8$$

IV. Again Nos. 3 and 5, but no white light (under conpensation point) pH 4.9

Light action H = +9.5 mm

30' 
$$x_{O2} = +45.6 \\ x_{CO2} = -53.0 \\ \gamma = -1.18$$
 
$$\frac{1}{\varphi} = \frac{30 \cdot 5.4}{45.6} = 3.6$$

V. Again No. 7, but with half light intensity (Actinometer, 2.75 μl O<sub>2</sub> per minute), p<sub>H</sub> 9.2.

10' red light — 24 ,, 10' dark — 28 ,, 10' red light — 24.5 ,, 10' red light — 27.0 ,, 10' red light — 23 ,, 
Light action 10' 27.5 — 23.8 = 
$$+$$
 3.7 mm = 2.42  $\mu$ l 
$$\frac{1}{\varphi} = \frac{10 \cdot 2.75}{2.42} = 11.3$$

The total duration of these experiments was 8 hours.

#### IX. SUMMARY AND CONCLUSION

Since development of the new methods and procedures described, in a sequence of thirty experimental days, almost without exception quantum efficiencies of 3 to 5 quanta per molecule of  $O_2$  produced by the action of red light have been obtained. The simultaneously observed quotients of  $\frac{CO_2}{O_2}$  for light action lay between —0.8 and —1.3, which means that the quantum efficiencies for  $CO_2$  consumption in red light were essentially the same as those for  $O_2$  production.

These results were obtained not only with low light intensities below the compen-Rejerences p. 346. sation point and for short periods of time (minutes), but also with light intensities well above the compensation point (several fold), and in experiments lasting many hours. It is important to emphasize that with the same cell suspension the same quantum yields may be obtained both below and far above the compensation point.

The new results resolve several uncertainties left open by the experiments of 1923. At that time the light intensities were so low that only a fraction of the respiration was compensated by the light. Thus the objection could never have been refuted that light inhibited respiration anticatalytically, that is, without expenditure of energy. But now, in the experiments above the compensation point, this question is eliminated, and chemical energy, corresponding to positive  $O_2$  production and  $CO_2$  consumption, is in fact clearly gained.

It was a further shortcoming of the experiments of 1923, that the yields had been determined only for short periods of time (e.g., 10 minutes). But now, in the experiments above the compensation point, the cells are so nearly under their natural culture conditions, that there is no evident time limit to yield determinations. Thermodynamically this is a noteworthy advance since the longer the experiments the surer becomes the necessary condition of all calculations of yield: that the absorbed light energy is the sole source of energy for the photosynthetic processes.

Finally, we may point out that the methodology has been so simplified that efficiency determinations can be carried out wherever simple manometric equipment and a suitable light source are available, without the need of a bolometer, thermopile, cathetometer or special differential manometer. In fact, demonstration of the high quantum efficiencies reported in this paper may readily be made in the laboratory classroom.

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#### RÉSUMÉ ET CONCLUSIONS

Depuis le développement des nouvelles méthodes et des nouveaux procédés décrits, nous avons trouvé, à peu près sans exception, une efficience de 3 à 5 quanta par molécule d'oxygène produite par l'action de la lumière rouge. Les coefficients  $\frac{\text{CO}_2}{\text{O}_2}$  observés simultanément pour l'action de la lumière se trouvaient entre —0.8 et —1.3, ce qui signifie que l'efficience en quanta pour la lumière rouge est à peu près la même pour la consommation de  $\text{CO}_3$  que pour la production de  $\text{O}_2$ .

Ces résultats ont été obtenus non seulement pour de faibles intensités et de courtes périodes, mais aussi pour des intensités bien au-dessus du point de compensation (plusieurs fois) et pour des expériences durant plusieurs heures. Il est intéressant de noter que l'on peut obtenir les mêmes rendements en quanta pour une même suspension cellulaire au-dessous et au-dessus du point de compensation.

Les nouveaux résultats résolvent plusieurs incertitudes qui avaient subsistées après les expériences de 1923. A cette époque, les intensités de lumière étaient si faibles que seule une fraction de

References p. 346.

la respiration était compensée par la lumière. C'est pourquoi, l'objection n'a jamais pu être réfutée sclon laquelle la lumière empêcherait la respiration anticatalytiquement, c.à.d. sans dépense d'énérgie. Actuellement cette question se trouve éliminée par les expériences au-dessus du points de compensation et on a vraiment un gain en energie chimique correspondant à une production positive de

 $O_2$  et une consommation de  $CO_2$ .

Une autre insuffisance des expériences de 1923 est due au fait que les rendements avaient été déterminés seulement pour des périodes brèves (p. ex. 10 minutes). Actuellement, où l'on travaille au dessus du points de compensation, les cellules se trouvent si près de leurs conditions de culture naturelles qu'il n'y a pas de temps limité évident pour les déterminations de rendement. C'est un sérieux avantage du point de vue thermodynamique, car plus les expériences sont longues, et plus sûrement la condition nécessaire pour toute détermination de rendement sera remplie, c.à.d. que la lumière absorbeé soit la seule source d'énérgie pour le processus photosynthétique.

Finalement, nous avons, tellement simplifié la méthodologie que des déterminations d'efficience simplifiées peuvent être effectuées facilement partout où l'on dispose d'un simple manomètre et d'une source de lumière adéquate. On n'a pas besoin de bolomètre, de thermopile, de cathétomètre, ni de manomètre différentiel spécial. En effet, l'on peut démontrer l'efficience quantique élevée, rapportée

dans ce mémoire, dans un laboratoire de classe.

#### ZUSAMMENFASSUNG UND SCHLUSSFOLGERUNGEN

Seit die hier beschriebenen neuen Methoden und Verfahren entwickelt worden sind, haben wir in einer Reihe von 30 Arbeitstagen fast ohne Ausnahme Quantumleistungen von 3 bis 5 Quanta pro Molekül  $O_2$  (gebildet unter der Einwirkung von rotem Licht) gefunden. Gleichzeitig wurden Quotienten  $\frac{CO_2}{O_2}$  für die Lichtwirkung gefunden, die zwischen —o.8 und —1.3 lagen; dies bedeutet dass

die Quantumleistung in rotem Licht für  $\mathrm{CO_2}$ -Aufnahme und  $\mathrm{O_2}$ -Abgabe ungefähr gleich war.

Diese Ergebnisse wurden nicht nur für niedrige, unter dem Kompensationspunkt gelegene Lichtintensitäten und für kurze Zeitspannen (Minuten) gefunden, sondern auch für hohe, weit über dem Kompensationspunkt gelegene Lichtintensitäten und für Versuche von mehreren Stunden. Mit der gleichen Zellsuspension kann man unter- und oberhalb des Kompensationspunktes dieselbe Quantumausbeute erhalten.

Die neuen Ergebnisse beheben einige Unsicherheiten der Versuche von 1923. Damals waren die Lichtintensitäten so gering, dass nur ein Teil der Atmung durch das Licht kompensiert wurde. Der Einwand, dass das Licht die Atmung antikatalytisch, also ohne Energieverbrauch hemme, konnte daher nie widerlegt werden. Nun aber, in den Versuchen oberhalb des Kompensationspunktes, ist diese Frage erledigt; es wird wirklich Energie entsprechend der Abgabe von O<sub>2</sub> und Aufnahme

von CO2 gewonnen.

Ein anderer Mangel der Versuche von 1923 bestand darin, dass die Ausbeuten nur über eine kurze Zeitspanne (z.B. 10 Minuten) bestimmt wurden. Nun aber, in den Versuchen oberhalb des Kompensationspunktes, befinden sich die Zellen so nahe den Bedingungen einer normalen Kultur, dass eine offensichtliche Zeitgrenze für Bestimmungen der Ausbeute nicht besteht. Thermodynamisch gesehen ist das ein wichtiger Fortschritt, denn je länger die Versuchszeit, desto sicherer wird die für alle Berechnungen der Ausbeute notwendige Bedingung erfüllt sein: dass nämlich die absorbierte Lichtenergie die einzige Energiequelle für den photosynthetischen Vorgang sei.

Endlich können wir darauf hinweisen, dass wir die Methodologie so vereinfacht haben, dass Leistungsbestimmungen mit einem einfachen Manometer und einer passenden Lichtquelle, ohne Bolometer, Thermoelement, Cathetometer und Spezial-Differentialmanometer ausgeführt werden können. So können die hier mitgeteilten hohen Quantumleistungen im Schullaboratorium nachge-

wiesen werden.

#### APPENDIX

I. Emerson has objected<sup>2,5</sup> to the yield determinations of 1923<sup>1</sup> and 1948<sup>4</sup> on the ground that the assimilatory  $\gamma = \text{CO}_2/\text{O}_2$  was not determined simultaneously with the yield  $\varphi$ ; *i.e.*, that the value of  $\gamma$  employed, —0.91, which had been determined gas analytically, may not be the  $\gamma$  during the different  $\gamma$ -determinations carried out for different periods of time, light intensities, and cell cultures.

As has been mentioned, we have observed experimental fluctuations of  $\gamma$  from -0.8 to -1.3 If we had used these  $\gamma$ -values in 1923 for the computation of q, let us see what the values of q would have been.

The volume of our vessel was 37.0 ml and the volume of the liquid phase 16.53 ml. For 10° C

$$\begin{split} k_{\text{CO}_2} &= 5.67 & k_{\text{O}_2} = \text{1.70} \\ K_{\text{O}_2} &= \frac{k_{\text{CO}_2} \cdot k_{\text{O}_2}}{k_{\text{CO}_2} + \gamma \ k_{\text{O}_2}} = \frac{5.67 \cdot \text{1.70}}{5.67 + \gamma \ \text{1.70}} \end{split}$$

Therefore

where the underlined values are the values used and obtained in 1923. This calculation shows that Emerson's objection was not very significant and could not explain the divergent quantum requirements of 4 against 10 to 12.

11. In an effort to avoid difficulties caused by fluctuations of γ. Emerson and Lewis made quantum-efficiency measurements in carbonate-bicarbonate solutions at p<sub>H</sub> 9.1, which kept the CO<sub>2</sub>-pressure constant instead of using culture medium at p<sub>H</sub> 4.9. They claimed<sup>5</sup> that in such alkaline solutions the quantum-efficiency was the same as in the acid culture medium: "then we find the yields measured in acid phosphate culture medium are in good agreement with those measured in carbonate mixture".

But the experimental data were not presented to substantiate this important statement. We can confirm Emerson's finding that in the carbonate-bicarbonate mixtures the quantum-requirement is 10 to 12, but we cannot confirm that the same quantum efficiency is obtained in the acid culture medium. Data presented in protocol 3 show that very different quantum-efficiencies are obtained if we determine the quantum efficiency of aliquot portions of a cell suspension in carbonate mixture at PH 9.1 and in culture medium at PH 5. The quantum values observed in the following time sequence were

|                                          | $\frac{1}{\varphi}$ |
|------------------------------------------|---------------------|
| In carbonate mixture at p <sub>H</sub> 9 | 10.5                |
| In culture medium at $p_{\rm H}$ 5       | 3.9                 |
| In carbonate mixture at p <sub>H</sub> 9 | 9.8                 |
| In culture medium at $p_{\mathrm{H}}$ 5  | 3.6                 |
| In carbonate mixture at p <sub>H</sub> 9 | 11.3                |

where the asterisked values were obtained above the compensation point and the others below the compensation point.

Maximum yields should therefore not be determined in the carbonate mixture, as has been done frequently during the last 10 years.

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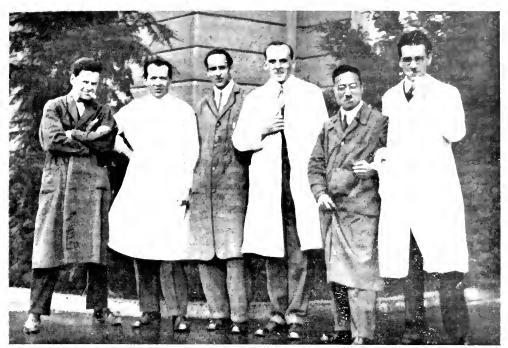


Fig. 1. Left to right: F. Lipmann, D. Nachmansohn, S. Ochoa, F. O. Schmitt, K. Iwasaki, P. Rothschild, Kaiser Wilhelm Institut für Biologie, Berlin Dahlem, 1928.



Fig. 2. Left to right: Sitting: O. Meyerhof and A. V. Hill, Starting K. L. Gerardo A. Muralt, G. Benetato, H. Blaschko, A. Grollman, H. Laser, Miss Wagner, W. Schulz, E. Boyland, Kaiser Wilhelm Institut für Medizinische Forschung, Heidelberg, 1931.

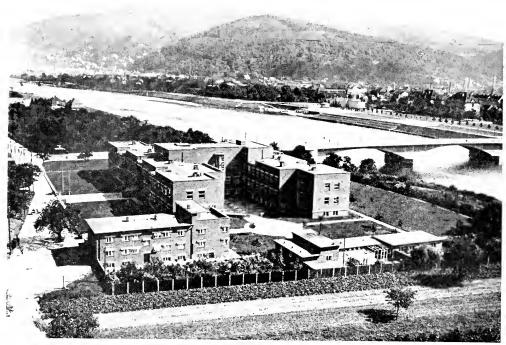


Fig. 3. Kaiser Wilhelm Institut für Medizinische Forschung, Heidelberg.



Fig. 4. Left to right: S. Korey, D. Nachmansohn, D. Burk, A. v. Szent-Györgyi, O. Warburg, O. Meyerhof, C. Neuberg, G. Wald.
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